The functional organisation of promyelocytic leukaemia nuclear bodies in human interphase cells

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Abstract

Promyelocytic leukaemia (PML) nuclear bodies are nuclear structures found in a variety of normal tissues and cell lines. They have been implicated in diverse human diseases. In particular, the major constituent, the PML protein, forms a fusion product with another protein in acute promyelocytic leukaemia (APL). These bodies however, also recruit over thirty different proteins with disparate functions. As such, no definite role of these bodies has been discovered, although proposed functions include gene transcription, cell cycle control and deoxyribonucleic acid (DNA) repair. This thesis describes the association of PML bodies with different genomic loci, using principally confocal microscopy and a novel statistical model. The aim was to use such associations to determine if a functional basis exists for the intranuclear pattern of PML bodies.

By analyzing loci-PML body distances for different gene loci, it was found that the distance between a locus and its nearest PML body correlates with the transcriptional activity and gene density around the locus. This was confirmed when regions of specific gene activation were examined. However, using RNA-FISH (ribonucleic acid-fluorescence *in situ* hybridisation) methodology and RNA interference (RNAi) knockdown studies, PML bodies were found not to be directly involved in gene transcription. Furthermore, cells in S-phase were examined in more detail, and it was found that PML bodies also associated statistically with actively replicating loci.

The experiments performed suggest a non-random and functional basis for the positioning of PML bodies. This thesis proposes that PML bodies are multifunctional structures that lie predominantly in nuclear compartments of high transcriptional activity, although they also associate with regions of DNA replication. Finally, this work strengthens the model of the nucleus as a highly organised structure.

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Abbreviations

Abbreviation	Full nomenclature
(d)G/A/C/T/U/NTP	(Deoxy)-guanine/adenosine/cytosine/thymine/uridine/ nucleotide triphosphate
°C	degree Celsius
3D	Three dimensional
aa	amino acid(s)
AP	alkaline phosphatase
APL	Acute promyelocytic leukaemia
AR	Androgen receptor
ARS	Autonomous replicating sequence
ATCC	American Type Culture Collection
ATPase	adenosine triphosphatase
ATRA	all-trans retinoic acid
BAC/PAC	bacterial/P-1 artificial chromosome
Bax	Bcl2-associated X protein
BLS	Bare lymphocyte syndrome
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic AMP
CB	Cajal (coiled) body
CBP	cAMP-response element binding protein
CCD	Cooled coupled devise
cdc	Cell division cycle
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CHO	Chinese hamster ovary
CIITA	Class II transactivator
CR	centiRays
CREB	cAMP Response Element Binding
CSK	Cytoskeletal (buffer)
CT	Chromosome territory
Ct	Calculated threshold
D	Dalton
DAPI	4'6'-diamino-2-phenylindole
DHFR	Dihydrofolate reductase
DMBA	dimethylbenzanthracine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRB	5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole)
DTT	dithiothreitol
EDC	Epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
EM	electron microscopy
EST	expressed sequence tag
FACS ®	registered trademark of Becton Dickinson and Company for a fluorescence-activated cell sorter

fc,dfc,gc	Fibrillar centre, dense fibrillar component, granular component
FCS	fetal calf serum
FGF	fibroblast growth factor
FISH	Fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FU	fluorouridine
GAS	gamma-activation sequence
G-band	Giemsa-band
GFP	green fluorescent protein
GM-CSF	Granulocyte/monocyte-colony stimulating factor
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
HAT	Histone acetyltranferase
HDAC	Histone deactylase
HIPK	Homeobox-interacting protein kinase
HLA	Human leukocyte antigen
HP1	Heterochromatin 1
HRP	horseradish peroxidase
IC	Initiation complex
ICD	Interchromatin domain
ICS	IFN consensus sequence
IdU	Iodo-deoxyuridine
IFNα/β/γ	interferon-alpha/beta/gamma
IFNγR	Interferon-gamma receptor
IGC	Interchromosomal granule cluster
IL	interleukin
IP	immunoprecipitation
IRF	Interferon response factor
ISRE	Interferon-stimulated response element
JAK	Janus kinase
JNK	Jun N-terminal kinase
kb	kilobase(s)
kD	kilodalton
KRAB	Kruppel-associated box
LINE	Long interspersed nuclear element
MARs	Matrix attachment (or associated) regions
Mb	megabase
MEF	Mouse embryonic fibroblast
MHC	major histocompatibility complex
mmd	mean minimum distance
mRNA	messenger RNA
MRS	MAR/SAR recognition sequence
n	number in a study or group
NA	numerical aperture
ND10	Nuclear dot-10
NER	Nucleotide excision repair
NFkB	Nuclear factor kappa-B
NHEJ	Non-homologous end joining
NK	Natural killer
NLS	nuclear localization signal
NP-40	Nonidet P-40

NPM	nucleophosmin
NS	not significant
NuMA	Nuclear mitotic apparatus
OD	optical density
ORC	Origin replication complex
PABL	Pseudoautosomal boundary-like sequence
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PF	Perichromatin fibril
PFA	paraformaldehyde
PHA	phytohaemagglutinin
PI	Propidium iodide
Pipes	[1,4-piperazinebis(ethane sulfonic acid)]
PML	promyelocytic leukaemia
POD	PML oncogenic domain
Pol	polymerase
PR	Progesterone receptor
R	correlation coefficient
RARα	Retinoic acid receptor-alpha
RARE	Retinoic acid response element
R-band	Reverse-band
RC	Replicative complex
RFP	Ret finger protein
RIDGE	Regions of increased gene expression
RING	Really interesting new gene
RNA	ribonucleic acid
RNAi	RNA interference
Rnase	ribonuclease
RNP	ribonucleoprotein
RPA	Replication protein A
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
RXR	Retinoid X receptor
SAGE	Serial analysis of gene expression
SARs	Scaffold attachment (or associated) regions
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFC	Splicing factor compartment
SINE	Short interspersed nuclear element
SiRNA	small-interfering RNA
Sn,sno,scaRNA	Small nuclear/small nucleolar/small CB-specific RNA
SSC	standard saline citrate
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
t test	Student's t test
TAE	Tris-acetate-EDTA
TAP/LMP	transporter associated with antigen processing/large multifunctional protease
TIF	Transcription intermediary factor
TIFF	Tagged image file
TNB	Tris-HCl, NaCl, BSA

TNFα	tumour necrosis factor-alpha
TNT	Tris-HCl, NaCl, Tween-20
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Texas red
TRIM	Tripartite motif
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TSA	Trichostatin A
UV	ultraviolet
Y2H	Yeast Two-Hybrid

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It was six men of Indostan, to learning much inclined,
who went to see the elephant (Though all of them were blind),
that each by observation, might satisfy his mind.

The first approached the elephant, and, happening to fall,
against his broad and sturdy side, at once began to bawl:
"God bless me! but the elephant, is nothing but a wall !"

The second feeling of the tusk, cried: "Ho! what have we here,
so very round and smooth and sharp? To me tis mighty clear,
this wonder of an elephant, is very like a spear!"

The third approached the animal, and, happening to take,
the squirming trunk within his hands, "I see," quoth he,
the elephant is very like a snake!"

The fourth reached out his eager hand, and felt about the knee:
"What most this wondrous beast is like, is mighty plain," quoth he;
"Tis clear enough the elephant is very like a tree."

The fifth, who chanced to touch the ear, Said; "E'en the blindest man
can tell what this resembles most; Deny the fact who can,
This marvel of an elephant, is very like a fan!"

The sixth no sooner had begun, about the beast to grope,
than, seizing on the swinging tail, that fell within his scope,
"I see," quothe he, "the elephant is very like a rope!"

And so these men of Indostan, disputed loud and long,
each in his own opinion, exceeding stiff and strong,
Though each was partly in the right, and all were in the wrong!

So, oft in theologic wars, the disputants, I ween,
tread on in utter ignorance, of what each other mean,
and prate about the elephant, not one of them has seen!

John Godfrey Saxe (1816 - 1887)
from the collection of "Best Loved Poems of The American People"

Origin:
Parable of the Blind Men and the Elephant
Udana 68-69: Jainism and Buddhism.

Chapter 1: Introduction

The nucleus is a membrane-bound organelle in the eukaryotic cell that contains the genome. The role of the nucleus is to provide an environment suitable for both expression and maintenance of the genome. This comprises a broad range of activities, including DNA replication, DNA damage sensing and repair, transcription, RNA processing and export.

The constituents of the nucleus during interphase can be divided into two broad categories: the DNA packaged as chromatin, and the other components (often proteins) involved in the biochemical processes. Both sets of constituents are recognised as having structural organisation (for recent reviews see Cremer and Cremer, 2001; Dundr and Misteli, 2001; Spector, 2003). Chromatin is divided into discrete chromosomes as visualised in metaphase, but also occupies distinct regions in the interphase nucleus. Variations in gene distribution within and between chromosomes suggest functional differences across the genome. Many proteins in the nucleus are also organised into visible structures or bodies, which are often located between chromosomes.

This structural subdivision shows that biological processes are partitioned into spatially and/or temporally discrete compartments, thus allowing for a much greater degree of complexity and efficiency than a simple system consisting of a homogenous mixture. However, the coordination of different processes in time and space within the nucleus remains to be fully understood. It is also not known how the structural organisation of chromatin and nuclear bodies is actually formed and maintained during interphase.

These questions have only begun to be addressed. Recent advances in cell biology techniques allow the analysis of both functional and structural aspects of the nucleus. In the past, light and electron microscope (EM) studies have concentrated on the structural components of the nucleus. More recently, the use of improved fluorescence techniques, three-dimensional (3D) confocal microscopy, and live cell imaging and *in vivo* analysis of nuclear dynamics have added a more functional element to nuclear research.

PML nuclear bodies have generated much interest since the finding that they are disrupted in acute promyelocytic leukaemia (de The *et al.*, 1991; Kakizuka *et al.*, 1991). However, the underlying function of these bodies is largely unknown. The aim of this

thesis is to elucidate the function(s) of PML nuclear bodies by analysing their spatial organisation and interactions with the genome.

1.1 The organisation of the genome

1.1.1 Structure of the chromosome

The human genome consists of 3.4 billion base pairs, and contains approximately 25,000 to 30,000 genes (Lander *et al.*, 2001; Venter *et al.*, 2001). Rather than the genes being interspersed at regular intervals along each chromosome, it is well recognised that gene density in humans and other higher eukaryotes varies in different genomic regions. In addition, these regions have variations in chromatin structure and DNA sequence composition, which may in turn indicate their different functional properties.

1.1.1.1 Chromosome bands and GC composition

The differences in the composition of the chromosomes can be inferred from the presence of distinctive bands along the length of the metaphase chromosome. These transverse bands were first visualised by specific DNA-binding stains, in some cases following various chemical treatments (reviewed in Verma and Babu, 1995). Subsequently, other banding techniques were developed. The banding patterns obtained by the various methods often correlate with each other, suggesting that bands arise from fundamental differences in properties of the chromatin. The common banding techniques are presented in Table 1.1.

The earliest studies of chromosome banding was performed by Caspersson *et al*, who used quinacrine mustard, a highly AT-specific fluorochrome, to detect the so-called Q-bands (Caspersson *et al.*, 1970). Subsequently, G-bands, identified by Giemsa staining following salt or trypsin treatment, were also found to highlight AT-rich regions. In contrast, R-bands (the Reverse of Q- or G-bands) are identified by pre-treatment with hot alkali to denature AT-rich DNA, leaving the more resistant GC-rich bands to be stained by Giemsa. Other stains that have a preference for GC- or AT-rich DNA also detect patterns similar to R- and G-bands.

Chromosome banding therefore reflects differences in nucleotide composition across the genome. However, the variations in base composition of adjacent bands are thought to be insufficient to explain the band patterns, as the GC content of G-bands is only

Table 1.1. Chromosome banding techniques.

Banding	Description
Q-banding	AT-specific fluorochromes: quinacrine, Hoechst 33258 or DAPI (6-diamidino-2-phenylindole)
G-banding	Giemsa staining after incubation with trypsin or salt solution at 60°C (similar to Q-banding)
R-banding	Giemsa staining after incubation with hot (80-90°C) alkali or acidic saline (giving a reverse pattern to G-banding)
Negative Q-banding	GC-specific fluorochromes: chromomycin actinomycin D and mithramycin (similar to R-banding)
C-banding	Giemsa staining of heterochromatin after acid/alkali treatment and incubation in hot SSC
Replication banding	Incorporation of BrdU during either early or late S-phase followed by Giemsa or antibody staining (see Section 1.1.2.4)
In situ hybridisation banding	Hybridisation of repeat sequences of DNA to produce patterns similar to G- or R-bands (see Section 1.1.1.4)
Antibody or immunocytogenetic banding	Binding of antibodies to specific chromatin or protein structures. Examples include antibodies detecting Z-DNA, 5-methylcytosine or acetylated histone H3 (see Section 1.1.2.1)

3.2% more than R-bands (Holmquist *et al.*, 1982). Laemmli and colleagues subsequently proposed that chromosome bands may also be related to the presence of specific ~200bp AT-rich sequences called scaffold-attachment regions (SARs) (Saitoh and Laemmli, 1993). EM studies show that during metaphase, chromatin is tethered via these SARs to a scaffold to form loop domains of 50-150 kilobases (kb) (Paulson and Laemmli, 1977). In G-bands, the backbone of SARs forms a so-called AT-queue that is tightly packed, with short DNA loops. In R-bands, the AT-queue is more loosely packed with larger DNA loops. Thus, R-bands, which have larger, more open chromatin loops, show higher endonuclease sensitivity and increased number of breakpoints (Kerem *et al.*, 1984).

1.1.1.2 Banding, isochores and gene density

Another view of the human genome, and generally that of other warm-blooded vertebrates, is derived by classifying blocks of DNA (of about 300 kb) according to their GC-richness, as proposed by Bernardi and colleagues (reviewed in Bernardi, 2000). DNA fragments from warm-blooded vertebrates were separated using density gradient caesium chloride centrifugation, GC-rich DNA being denser that AT-rich DNA (Macaya *et al.*, 1976). Five classes or 'isochores' were isolated in order of GC-richness (Bernardi, 1989): L1 (<38% GC), L2 (~40%), H1 (~45%), H2 (~50%) and H3 (~53%). An additional fraction consisted of satellite and ribosomal DNA.

As expected from the GC composition, the different isochores generally correlate with banding patterns (Saccone *et al.*, 1999). DNA from the H3 isochore, and from some of the H1 and H2 isochores, hybridises to T-bands (a subset of R-bands with the highest GC content), while DNA from the L1 and L2 isochores (with a minor component of H1) hybridises to G-bands. The R-bands are more heterogeneous, with the majority component being DNA from the H1 and L1/2 (but with some H2 and H3) isochores. In addition, both isochores and banding patterns correlate with the gene density in the different regions, with a general decrease in gene density from the H3 to the L1 isochore (Zoubak *et al.*, 1996). The H3 isochore accounts for only 3% of the genome yet contains at least 16 times the number of genes found in the L isochores, which account for 62.9% of the genome (Mouchiroud *et al.*, 1991). Thus, the majority of genes (both housekeeping and tissue-specific) map to R- and T-bands, while only ~20% of mapped genes map to G-bands(Craig and Bickmore, 1993; Holmquist, 1992).

1.1.1.3 Repetitive sequences

The discussion so far has dealt with coding regions of the chromosomes. Another fraction of the genome contains satellite or tandemly repetitive sequences, which may be classified as highly or middle repeated sequences (Bernardi, 1989). Most highly repeated sequences are large arrays of satellite DNA found at the centromeres of the chromosomes, and thus often correspond to the nuclease resistant C-bands (Yunis *et al.*, 1971). They form the constitutive heterochromatin (see below) present throughout the life of the organism.

In addition to satellite repeats, eukaryotic genomes also contain a large number of interspersed repeat units that have spread between chromosomes by a retrotransposition process. The sequences and include short and long interspersed nuclear elements (SINEs and LINEs), found in R-bands and G-bands respectively (Korenberg and Rykowski, 1988). The major human SINE is the ~300bp GC-rich (56% GC) *Alu* family, which accounts for 10% of the human genome and is located within introns of genes and between genes (reviewed in Kreahling and Graveley, 2004). These sequences can alter the expression or splicing of genes, and may have contributed to disease states or evolutionary potential (Puget *et al.*, 1999; Sorek *et al.*, 2002).

1.1.2 Functional organisation of the chromosome

The sections above have dealt largely with variations in the DNA composition of the genome. However, it is known that variations in the structure and epigenetic modification of chromatin also occur in interphase to regulate the activities of the different genomic regions.

1.1.2.1 Euchromatin and heterochromatin

Electron microscopic studies have shown that the interphase nucleus is not uniform in density. Clumps of densely packaged chromatin, termed heterochromatin, are interspersed between decondensed (from mitosis) euchromatin. Euchromatin is transcriptionally active or permissive, and is characterised by a more open chromatin conformation sensitive to DNase I digestion (Babu and Verma, 1987). It includes both R- and G-bands.

Heterochromatin can be further classified into facultative or constitutive heterochromatin. Facultative heterochromatin contains coding sequences, but the genes

are silenced during development and tissue differentiation, such as in the inactive X chromosome in female somatic cells (reviewed in Heard *et al.*, 1997). This highly condensed chromatin is thought to prevent binding of transcription factors. Facultative heterochromatin is found in G-bands. In contrast, constitutive heterochromatin, found in C-bands, consists of mostly satellite DNA, and does not contain coding sequences. Rather than being transcriptionally inert, it may play an active role in regulating transcription. Brown *et al* showed that in differentiating B-lymphocytes, the silencing of certain genes is mediated by the Ikaros protein family, which targets these genes to regions of centromeric heterochromatin within the nucleus (Brown *et al.*, 2001; Brown *et al.*, 1997).

1.1.2.2 Histone modification and DNA methylation

In the nucleus, chromatin is packaged into nucleosomes by the coiling of DNA around an octet of histone proteins, resulting in a 'string of beads' conformation. Chromatin (de)condensation is thought to result from the modification of these core histones. Several forms of covalent modification of specific amino acids contribute to this process. Of these, the most widely studied is the lysine acetylation of the amino terminus of histones H3 and H4, which has been associated with active chromatin (reviewed in Kurdistani and Grunstein, 2003). Specific histone acetyltransferases (HATs) direct this process, leading to a conformation of the nucleosome that allows binding of general transcription factors. Histones are deacetylated by histone deacetylases (HDACs), and these complexes can recruit transcriptional corepressors (Nagy *et al.*, 1997). Fluorescence *in situ* hybridisation (FISH) analysis, using nucleosome-derived DNA as probes, shows patterns of histone acetylation on human chromosomes similar to chromosome bands. DNA from hypoacetylated chromatin labels several pericentric regions, whereas DNA from highly acetylated chromatin labels R-bands (Breneman *et al.*, 1996). Although visualised on metaphase chromosomes, histone acetylation states persist in the interphase chromatin. Other covalent modifications of histones include lysine methylation, arginine methylation, lysine ubiquitination and serine phosphorylation (reviewed in Khorasanizadeh, 2004).

Another epigenetic mechanism for control of gene transcription is DNA methylation. It involves the addition of methyl groups to the cytosine in CpG dinucleotides. Specific dense regions of CpG (more than 200 bp) are found at the 5' end of many mammalian genes, termed CpG islands (Ioshikhes and Zhang, 2000). It is estimated that around

60% of human genes, with all housekeeping and 40% of tissue-specific genes, possess most of the CpG islands in the genome (Larsen *et al.*, 1992). The role of DNA methylation is to repress transcription during development and differentiation of specific tissues, enabling tissue-specific expression. Methylation represses genes by recruiting methyl-CpG binding proteins to the CpG dinucleotides (Fuks *et al.*, 2003). This in turn mediates histone deacetylation and methylation. In the blastula stage of embryonic development, most of the DNA is unmethylated but as differentiation begins, a wave of methylation modifies most of the genome except for CpG islands. The CpG islands of tissue-specific genes are unmethylated only in tissues where the genes are expressed (reviewed in Kass *et al.*, 1997). In the genome of a mammalian adult, 60-90% of these CpG dinucleotides are methylated (Bird, 1986). Craig and Bickmore (Craig and Bickmore, 1994) used FISH to map CpG islands on human metaphase chromosomes. They showed that >80% of CpG islands map to R-bands and of these, most appear to map to the T-bands. Thus, the frequency of CpG islands is also increased in GC-rich isochores (Aissani and Bernardi, 1991).

1.1.2.3 Chromosomes and transcriptional activity

Analysis of DNA methylation and histone modification maps suggests there are variations in both gene density and gene expression in different genomic regions. The technique Serial Analysis of Gene Expression (SAGE) uses short transcript tags to analyze transcriptional activity genome-wide (Velculescu *et al.*, 1995). Using this approach, over 18,000 10 bp-length tags from transcripts were used to create a Human Transcriptome genomic map for a variety of normal and tumour samples (Caron *et al.*, 2001). A broad correlation was found between gene density and the semi-quantitative expression levels of genes within the different regions. Regions of IncreaseD Gene Expression (RIDGEs) were found in gene-rich regions, including the Major Histocompatibility Complex (MHC) on chromosome 6 and regions in both arms of chromosome 19. More recently, these RIDGEs have been found to correspond to GC-rich, SINE-dense regions (Versteeg *et al.*, 2003). This agrees with a previous report which suggested that the gene-rich H3 isochore, which mainly maps to T-bands, is enriched in housekeeping genes (Bernardi, 1993).

1.1.2.4 Chromosomes and replication timing

The replication of the chromosome in S-phase of the cell cycle is a temporally coordinated event, lasting usually 6-8 hours in human cell lines (Kapp and Painter,

1977). At the chromatin level, replication initiates at multiple sites, termed replication origins. The region replicated from one origin is termed a replicon. Different origins are triggered at different times in S-phase, although origins are thought to be activated synchronously in clusters (Blumenthal *et al.*, 1974).

This variation in replication timing has been exploited to produce replication banding patterns. Cells are treated with various blocking agents to synchronise them in early or late S-phase, then incubated with bromodeoxyuridine (BrdU) and its incorporation into the chromosomes visualised with specific anti-BrdU antibodies (Vogel *et al.*, 1989). This produces either an early- or a late-banding pattern. It has been found that in general, T- and R-bands replicate in early S-phase, while G- and C-bands replicate in late S-phase (Holmquist *et al.*, 1982).

This also suggests that different isochores will replicate at different stages. Federico *et al* hybridised the GC-rich H3 isochore to metaphase chromosomes showing replication bands, and found it localised mainly in early replicating bands (Federico *et al.*, 1998). However, replication timing may be a function not of GC content, but of gene density and transcription (Bernardi, 1989). See Chapter 5 for further functional considerations of replication timing.

We can therefore see that, although the association between structural and functional properties of the different regions of the chromosomes is not exact, there is a broad division between regions which are active, and those which are less so. This is illustrated in Table 1.2, which describes properties of G- and R-bands.

1.2 Nuclear compartmentalization and function

The introduction thus far deals with the linear arrangement of genes on metaphase chromosomes. Structural and functional distributions of the genes during interphase have also been investigated within the 3D confines of the interphase nucleus. Many early studies focused on chromosomes, or on each of the non-chromatin nuclear structures in isolation. Increasingly, it is recognised that interactions between the chromosomes and nuclear bodies are functionally important. For the purposes of this introduction, the different nuclear structures will be described individually, but interactions between them will be mentioned where appropriate.

Table 1.2. Structural and functional properties of G- and R-bands.

G-bands	R-bands
GC-poor isochores L (+ H1)	GC-rich isochores H1,2,3 (+ L)
DNase I insensitive	DNase I sensitive
Few breakpoints/rearrangements	Higher number of breakpoints/rearrangements
LINE-rich	SINE-rich
Low gene density	High gene density
Low level of histone acetylation	High level of histone acetylation
Low number of CpG islands	Higher number of CpG islands
Tissue specific expression	House-keeping & tissue specific expression
Late replicating	Early replicating

1.2.1 Chromatin in interphase cells

In humans, the genome is several billion basepairs or approximately 6 feet long, and must be tightly packaged into a nucleus of several micrometres in diameter, yet be readily available both for replication and transcription in a coordinated manner. Such organisation includes the distribution of the different chromosomes in interphase, as well as the 3-dimensional organisation of the genes 'folded' within each chromosome.

1.2.1.1 Chromosome territories

More than one hundred years ago, it was suggested by Rabl and Boveri that chromatin was not diffused throughout the nucleus, but rather occupied distinct territories (Figure 1.1.A, for review see Spector, 2003). However, it was only within the last few decades that experiments have been able to support these claims. For example, ultraviolet irradiation of small regions of interphase Chinese hamster cells damaged specific chromosomal regions visualised in metaphase (Cremer *et al.*, 1982). Subsequently, interphase chromosomes were directly visualised in interspecies hybrid cell lines, where single human chromosomes were introduced into mouse nuclei. *In situ* hybridisation with total human DNA as a probe showed that this single chromosome occupied a discrete area of the nucleus (Manuelidis, 1985; Schardin *et al.*, 1985). This observation was confirmed using the FISH technique of "chromosome painting", where individual chromosomes were specifically labelled in interphase nuclei (Lichter *et al.*, 1988).

The space occupied by the chromosome was termed a (chromosome) territory. Between and even within these chromosome territories (CTs) is a chromatin-free space, initially termed the interchromosome domain, later termed the interchromatin domain (ICD) (Zirbel *et al.*, 1993). The ICD forms a network of channels of varying widths, extending to the nuclear pores, and contains nuclear bodies and smaller diffusible nuclear factors (Bridger *et al.*, 1998). These interchromosomal channels allow access to the nuclear interior and are a convenient means of transporting transcription products to the nuclear pores. It has been proposed that the interface between the surface of the CTs and the ICD is the major site of nuclear activity. Computer enhanced reconstruction of chromosome territories has suggested that chromosome territories are not smooth structures (Eils *et al.*, 1996). Studies on the topology of the active and inactive X chromosome territories showed that the active X territory had a more irregular surface, presumably allowing improved access to transcription machinery. Furthermore, it has been found that chromosome territories do not form impenetrable barriers. Introduction

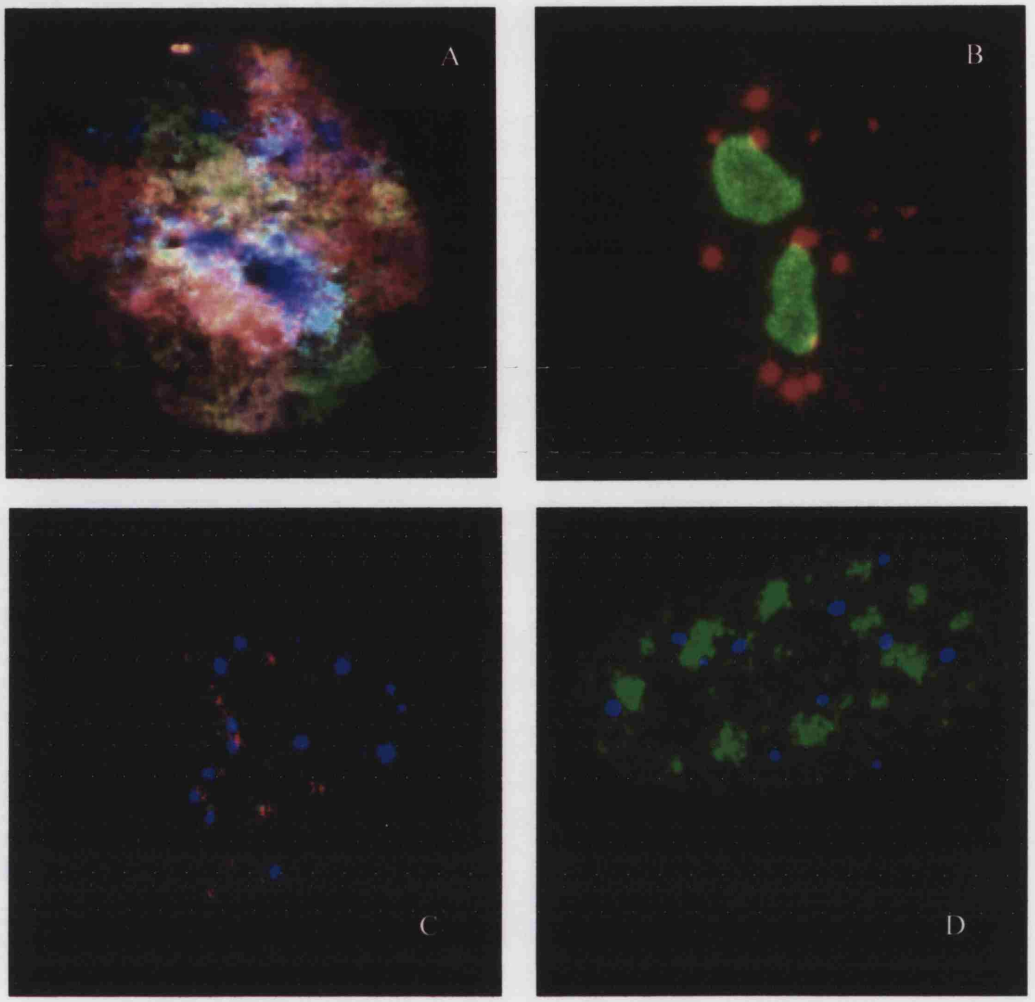

Figure 1.1 Fluorescent staining of nuclear compartments.
A: Multiplex-FISH (M-FISH) image of a human B cell interphase nucleus, showing discrete chromosome territories. B: Human fibroblast nucleus immunostained for nucleoli (green) and PML bodies (red). C: HeLa cell line immunostained with PML bodies (blue) and Cajal bodies (red). D: Human fibroblast nucleus immunostained for PML bodies (blue) and speckles/SFC using anti-SC-35 antibody (green). Images (A) courtesy of R.Vatcheva and (C) courtesy of C Shiels.

of fluorochrome-labelled dextrans into cell nuclei showed that molecules up to 580 kDa diffused freely in the nucleus (Lukacs *et al.*, 2000).

At present, there is still no agreement about whether chromosomes are arranged in any specific order in the nucleus. Nagele *et al* suggested that chromosomes in mice show highly ordered radial arrangements in the prometaphase rosette, which are reproducible over several cell cycles suggesting that their relative position in the interphase cell was also ordered (Nagele *et al.*, 1995). However, another study has showed that chromosomes in metaphase did not show any significant ordering (Allison and Nestor, 1999). It may be that the degree of order is cell-type specific (Chandley *et al.*, 1996). Ordering of chromosomes may also be species-specific. The chicken genome, for example, is contained in macro- and microchromosomes, with different radial arrangements during interphase (Habermann *et al.*, 2001). A recent study using live cell microscopy with labelled chromosome regions showed that during mitosis, the migration of chromosome regions tended to follow defined paths (Gerlich *et al.*, 2003). It was proposed that daughter cells have arrangements of chromosome territories that appear in parallel or mirror images to each other, and to the mother cell. Another study using a different cell line and methodology (photobleaching of specific nuclear regions) showed reorganisation of chromosome territories in daughter cells (Walter *et al.*, 2003). However, both studies agreed that positioning of whole territories is stable during most of the interphase, although some plasticity was observed in early G1-phase. This observation was also made using fluorochrome labelled DNA, which after several cell divisions allowed individual chromosome territories to be visualised in living cells. Using this technique, Cremer and colleagues revealed subtle changes in the shape and position of these CTs during interphase, although they remain static as a whole (Edelmann *et al.*, 2001).

Correlations of chromosome positioning with certain structural features have also been attempted. One study suggested that smaller chromosomes tend to be positioned more towards the nuclear interior (Sun *et al.*, 2000). However, later studies showed that gene density is a more accurate determinant of radial positioning of chromosomes (Croft *et al.*, 1999). For example, human chromosomes 18 and 19 are similar in genetic size, but vary in the number of genes they carry. Chromosome 18 is often found in the nuclear periphery, while chromosome 19 is more internal. This arrangement is plastic, with quiescent cells showing a relocation of chromosome 18 into the interior, which is

reversible, suggesting that chromosomal positioning is related to cellular activity. This correlation is seen in many different cell types (Cremer *et al.*, 2003; Cremer *et al.*, 2001), and is applicable to both spherical and flat nuclei.

Another issue is whether specific chromosomes are preferentially associated with each other. Associations exist between homologous chromosomes in interphase in Drosophilia diploid somatic cells (Henikoff, 1997). In human cells, such homologous associations are occasionally seen for gene-dense, smaller chromosomes, but it must be noted that this could be due to their preference for the nuclear interior (LaSalle and Lalande, 1996). It remains to be seen if such pairings are due to physical constraints within the nucleus.

Studies have also addressed the mechanism by which chromosome positions are maintained. One possibility is that certain CTs might attach directly to the nuclear envelope or lamina. Emerin is a protein found in the inner nuclear envelope, and mutations of the emerin gene are responsible for X-linked Emery-Dreifuss muscular dystrophy. Examination of lymphocytes from a patient with this syndrome showed no spatial differences in the territories from normal lymphocytes (Boyle *et al.*, 2001). Thus attachment of chromatin to the protein emerin was excluded as the mechanism for maintaining the positions of CTs.

Berezney and colleagues have suggested that CTs owe their structural integrity to an underlying nuclear matrix (which is analogous to the scaffold in metaphase chromosomes) containing protein and RNA (see Section 1.2.2.6). Disruption of this matrix by RNase and salt treatment leads to loss of the integrity of chromosome territories (Ma *et al.*, 1999). The use of increasing concentrations of salt also showed that chromosome 18 was released from the nuclear remnants prior to chromosome 19, suggesting that chromosomes with different gene densities form different associations with the nuclear matrix (Croft *et al.*, 1999).

The role of gene activity in maintaining chromosomal integrity has also been studied. Mouse cells were treated with Trichostatin A (TSA), a HDAC inhibitor, for up to five days, resulting in centromeres and heterochromatic regions being relocated from the nuclear interior to the periphery, accompanied by loss of heterochromatin 1 (HP1) proteins (Taddei *et al.*, 2001). This effect was thought to require several cell divisions,

and was shown to be reversible upon removal of TSA. Treatment of cells with transcriptional inhibitors also results in dispersal of euchromatic chromosome domains and whole chromosome territories (Haaf and Ward, 1996). Examination of chromosomes 18 and 19 in cells treated with transcriptional inhibitors revealed changes in the compaction of the chromosomes, but not their nuclear locations (Croft *et al.*, 1999).

1.2.1.2 Gene positioning within chromosome territories

In addition to the positions of whole chromosome territories, there is also much research towards understanding the organisation of the genomic regions within each territory. Rabl postulated, again more than a hundred years ago, that telomeres were attached to the nuclear envelope on one side of the nucleus, while the centromeres were attached on the opposite side. In 1934, McClintock discovered that densely stained chromosomal regions were related to the number and type of nucleoli in mutant strains of maize (for review see Hernandez-Verdun *et al.*, 2002). These regions were termed nucleolar-organising elements, bodies or regions (NORs). These were subsequently found to contain rDNA genes, which become transcriptionally active in early interphase and subsequently fuse to form the nucleoli.

Late-replicating, gene-poor regions were noted in subsequent reports to be located at the nuclear periphery and around the nucleolus compared with early-replicating, gene-rich regions (corresponding to R/T chromosome bands in metaphase chromosomes), which are found throughout the nuclear interior (Sadoni *et al.*, 1999). It has therefore been proposed that CTs may be "polar". More specific studies using probes targeting the centromeres, telomeres and individual gene loci have been used. An earlier study suggested that a looped structure is present in the inactive X chromosome, whereby the two telomeres associate with each other to form a horseshoe-like pattern (Walker *et al.*, 1991). Although this proposed looped structure was subsequently disputed (Dietzel *et al.*, 1998), some degree of folding of various chromosomes has been noted (Amrichova *et al.*, 2003).

Within in each chromosome territory, the GC-rich isochore H3 is more likely to be found at the territory periphery, while the gene-poor L isochores are found in the interior (Tajbakhsh *et al.*, 2000). Specific genes are also found to be arranged functionally, with certain active genes and clusters located on the external surfaces

(Kurz *et al.*, 1996). The relationship with transcription has been shown by Cremer and colleagues (Dietzel *et al.*, 1999). By analysing two genes, *ANT2* and *ANT3*, on the active and inactive X chromosomes, they found that both of the *ANT3* homologues (which are from pseudoautosomal regions of the X chromosome and are co-expressed), and the active copy on *ANT2* were present near the territory periphery. In contrast, the inactive *ANT2* gene was found more internal to its territory. Genes can also lie outside the territory as defined by whole chromosome painting forming large-scale extensions or "loops" (not to be confused with the DNA loops attached to the scaffold). This was initially found for the gene-rich Major Histocompatibility Complex (MHC) spanning several megabases in cells expressing MHC genes (Volpi *et al.*, 2000). Similar loops were subsequently noted for the Epidermal Differentiation Complex (EDC) on chromosome 1 in differentiating keratinocytes(Williams *et al.*, 2002). Other chromosomal regions that 'loop' out of their territories have been reported, and this configuration attributed to their gene density and transcriptional activity (Mahy *et al.*, 2002a).

Although these chromatin configurations demonstrated the positioning of active genes on the CT surface, transcription has been shown to take place throughout territories (Mahy *et al.*, 2002b; Verschure *et al.*, 1999). Furthermore, Verchure *et al* found that chromosome territories are not uniform in density, but rather have subdomains of more compact chromatin, linked by strands of chromatin material. Transcription foci were predominantly found at the surfaces of these compact chromatin domains, but *within* the territories. Similarly, Visser *et al* established that replication foci are distributed throughout entire chromosome territories in both early and late S-phase (Visser *et al.*, 1998). Thus, DNA metabolism takes place throughout the chromosome territory and is not confined to the surface as previously thought. This may be explained partly by the fact that the surface of chromosome territories does not form an impenetrable barrier, or that deep folds exist in the territories (Lukacs *et al.*, 2000).

Particular genes have also been shown to associate with specific nuclear compartments under certain conditions. As previously mentioned, several silent genes were found to associate with centromeric heterochromatin (Brown *et al.*, 2001; Brown *et al.*, 1997). Transcriptional silencing may also be controlled by positioning genes near the nuclear periphery, enabling interactions with the nuclear lamina (Nili *et al.*, 2001). Conversely, active genes associate with nuclear structures such as SC-35 domains, Cajal bodies, or

PML bodies (see Sections 1.2.2 and 3.1). A statistically increased association of certain gene loci with others has been reported. These studies suggest that genes are often found close to their translocation partners. An example is the spatial proximity of the *myc* gene on chromosome 8 with the immunoglobulin-heavy and -light chain genes on chromosomes 2, 14 or 22 (translocated in Burkitt's lymphoma and B-cell leukaemia) (Roix *et al.*, 2003).

1.2.1.3 Chromatin dynamics using live cell imaging

The studies described thus far have largely been performed in fixed cells and so are uninformative about chromosome movements *in vivo*. Live cell studies have shown that chromosome territories may have limited mobility (see Section 1.2.1.1). Specific loci can also be directly visualised in live cells, using lac-repressor protein fused to Green Fluorescent Protein (GFP) (Robinett *et al.*, 1996). This tagged protein labels stably-transfected lac operator repeat arrays in different chromosome locations. Early studies using this system on Chinese hamster ovary (CHO) cells allows decondensation of chromatin regions to be visualised in response to activator binding, which correlated to histone acetylation (Tumbar *et al.*, 1999). Although transcription was not involved in decondensation in this study, a subsequent study using a different reporter system showed that transcription was required (Muller *et al.*, 2001). More recently, Chen *et al* suggested that, at least for rDNA loci in the nucleolus, the binding of Upstream Binding Factor, a co-factor of RNA polymerase (pol) I, may induce a transcriptionally permissive state by chromatin decondensation (Chen *et al.*, 2004). Specific movements of loci were also noted. For example, one study showed the movement of a lac array from a peripheral to a more interior nuclear position upon VP16 viral protein targeting (Tumbar and Belmont, 2001).

The range of movements has also been analysed to determine if a mathematical model could be applied. Experiments in Drosophila and yeast showed that genes are mobile but appear constrained either by nuclear regions or chromosome territories (Heun *et al.*, 2001; Vazquez *et al.*, 2001). More recently, similar findings have been made in human cells, where movements of under 2 μm were noted, depending on the genomic region (Chubb *et al.*, 2002). It was found that loci near the nucleoli or at the nuclear periphery exhibited less mobility, suggesting structural attachments. The form of movement described suggested random Brownian motion of chromatin. However, directed movements of loci can be seen with VP16 as mentioned above. This movement

suggests a repositioning of loci into the interior of the nucleus. It remains to be seen if loci can actually be directed to specific sites or bodies.

1.2.2 Nuclear Bodies

Nuclear bodies, unlike the compartments (organelles) found within the cytosol, are not bounded by membranes and are more poorly delineated. As a result, only a few nuclear bodies have been extensively characterised functionally, the remainder having only a simple morphological or behavioural description. Of these, the best characterised are the nucleolus, Cajal bodies, speckles or splicing factor compartments (SFC) and PML bodies (see Figures 1.1.B, C, and D). PML bodies will be dealt in more detail in Section 1.3.

1.2.2.1 The nucleolus

This is one of the first and most prominent subnuclear structures to be described. It is dynamic, its size and organisation depending on cell type and status. The main function of the nucleolus is the biosynthesis of ribosomes. As mentioned above, the nucleolus is assembled around NORs, comprising tandemly repeated clusters of ribosomal RNA genes (rDNA) present in human on chromosomes 13, 14, 15, 21 and 22 (Hernandez-Verdun *et al.*, 2002). It is believed that heterochromatin at the NORs plays a role in nucleolar assembly, possibly via clustering of silent regions adjacent to active rDNA genes. The formation of nucleoli is related temporally to the cell cycle. Disassembly of nucleoli occurs in prophase, and reassembly occurs in late anaphase. This coincides with the repression and reactivation of RNA pol I transcription (Sirri *et al.*, 2000).

In a HeLa cell nucleus, only 120-150 of the rDNA genes are estimated to be active out of a total 540 genes (Jackson *et al.*, 1998). However, the transcription output accounts for 50-80% of the total transcriptional activity of the cell (Warner *et al.*, 2001). Each active gene has 100-120 RNA pol I protein molecules associated with it (Jackson *et al.*, 1998). The nucleolus consists of three distinct morphological regions through which rRNA biogenesis occurs in a vectorial manner: the "fibrillar centre" (*fc*), the surrounding "dense fibrillar component" (*dfc*), and the external "granular component" (*gc*). The *fc* consists of RNA pol I and its co-factors (Jordan *et al.*, 1996), active genes having a more peripheral localisation that extends into the *dfc* (Thiry *et al.*, 1988). rRNA primary transcripts transiently accumulate in the *dfc* and undergo a complex series of processing events, as they radiate out into the *gc* (Hozak *et al.*, 1994). The

ribosomal subunits are then exported into the cytoplasm, where final assembly of the ribosomes occurs.

In addition to being the site of rRNA synthesis and processing, the nucleolus is believed to be the site of assembly and action of other RNP complexes, consisting of small nuclear (nucleolar) RNAs (snRNAs/snoRNAs), many of which have a role in protein synthesis (Pederson and Politz, 2000). In yeast, the nucleolus also plays a role in the processing of small transcripts suggested to be tRNA, generated by RNA pol III (Bertrand *et al.*, 1998).

Recently, Lamond and colleagues have used a proteomic approach to determine the components of the nucleolus (Leung *et al.*, 2003). It was found that up to 30% of proteins were novel and uncharacterised. Furthermore, several proteins associate transiently or conditionally with nucleoli. It suggests that the nucleolus may serve as a depot in which proteins accumulate under specific conditions. For example, the nucleolus plays such a regulatory role for the p53 tumour suppressor protein. MDM2 is a protein that binds to p53 to target it for degradation. The activity of MDM2 itself is regulated by p19ARF, which sequesters it to the nucleolus (Weber *et al.*, 1999). This also results in the accumulation of p53 in the nucleolus (Zhang and Xiong, 1999). A DNA helicase involved in genomic stability, the Bloom syndrome protein BLM, is found in the nucleolus during S-phase (Yankiwski *et al.*, 2000). BLM, MDM2 and p53 are also found in PML bodies and will be discussed in more detail later (see Section 1.3.7).

1.2.2.2 Cajal bodies

Cajal bodies (CBs), previously known as "coiled bodies", were re-named after their discoverer, Santiago Ramon y Cajal who described them as "nucleolar accessory bodies" just over a hundred years ago (for review see Ogg and Lamond, 2002). They are highly conserved, being found in both plant and animal cells, suggesting they are involved in fundamental nuclear processes. A typical nucleus has 1-10 CBs although this number varies between cell types and within the cell cycle, being increased in late G1 and S-phases (Liu *et al.*, 2000). They are spherical in shape, having a diameter of 0.1-2 μm, and are highly dynamic, most likely moving by simple diffusion, although ATP dependent associations with genes have been noted (see below) (Platani *et al.*, 2002).

By EM, CBs appear as a tangle of coiled fibrillar strands (Monneron and Bernhard, 1969; Raska *et al.*, 1991). They are also detected by specific antibodies to the protein p80-coilin (antibodies to coilin were initially discovered in human autoimmune sera), although only a minor fraction of nuclear coilin is present in CBs at any given time (Andrade *et al.*, 1991). CBs are also enriched in spliceosomal snRNPs, several nucleolar proteins and snoRNPs, RNA pol II transcription factors (TFIIF and TFIIH), and a novel class of CB-specific small RNAs (scaRNAs) (Gall *et al.*, 1999). They do not contain DNA (Thiry, 1994).

The function of CBs remains unclear. They were initially found to be associated with the histone gene cluster in the lampbrush chromosomes of *Xenopus* (Callan *et al.*, 1991). They were subsequently found associated with chromosomal loci encoding histones and several sn- and sno- RNAs, U1, U2 and U3, in mammalian cells (Frey and Matera, 1995). This association appears to be dependent on the transcriptional activity of the genes (Jacobs *et al.*, 1999). While CBs are themselves unlikely to be sites of transcription or splicing (Ogg and Lamond, 2002), their association with these loci is suggested to be required for initiation of transcription (Shopland *et al.*, 2001). The finding that many subunits of RNA pol I, II and III, as well as splicing factors are found in CBs, suggests a role for CBs in the assembly of transcription factories (Gall *et al.*, 1999). The link between transcription and CBs is further illustrated by the fact that CBs do not form in cells when general transcription is inhibited in early G1 (Ferreira *et al.*, 1994).

There is increasing evidence that CBs are also functionally and spatially related to the nucleolus. Under normal circumstances, GFP-tagged CBs are seen to come into contact with the nucleoli (Boudonck *et al.*, 1999). Mutations of the coilin protein, transcriptional inhibition, and treatment with protein phosphatase inhibitor, okadaic acid, results in relocation of CBs to the periphery and interior of nucleoli (Raska *et al.*, 1990; Sleeman *et al.*, 1998). This may be related to the proposed function of CBs in co-ordinating the assembly and maturation of snRNPs. Following transcription of sn(o)RNA, many transcripts leave the nucleus, and are processed in the SMN/gemin complex in the cytoplasm (Carvalho *et al.*, 1999). The partially assembled sn(o)RNPs re-enter the nucleus, where they associate transiently with CBs before ending in the nucleoli or speckles (Gall *et al.*, 1999; Narayanan *et al.*, 1999). This suggests either that

CBs are involved in two distinct steps in sn(o)RNP biosynthesis (transcription and post-transcriptional modification) or that a feedback system exists (Matera, 1998).

1.2.2.3 Splicing factor compartments/Speckles and Perichromatin fibrils

Splicing factor compartments (SFCs) or speckles were initially discovered by immunofluorescence using serum from patients with autoimmune diseases (Beck, 1961). They contain various components of the pre-mRNA splicing machinery, including snRNPs, spliceosome subunits and protein factors (Spector *et al.*, 1991). In addition, they contain a hyperphosphorylated form of RNA polymerase II (RNA pol IIo), which is both an elongation and an inactive form (Bregman *et al.*, 1995). They are visualised under EM as interchromatin granule clusters (IGC), consisting of 0.5-1.0 μm domains containing granules of 20-25nm diameter (Swift, 1959). Antibodies against SFCs have also been found to label perichromatin fibrils (PF), which are structures distinct from IGCs under EM, consisting of fibres of variable thickness, containing nascent transcripts (Fakan, 1976). Some groups consider PFs and IGCs as two structural variants of SFCs (Smith *et al.*, 1999), while others consider PFs as distinct entities, located in the periphery of IGCs (Cmarko *et al.*, 1999). Some authors have named the PFs and IGCs together as SC-35 domains, after the protein splicing factor contained within both entities (Smith *et al.*, 1999).

Although the positions of SFCs are relatively static during interphase, their sizes and shapes are affected by transcription. Inhibition of transcription results in larger, or rounded structures (Spector *et al.*, 1983), while increased transcriptional states result in weaker staining, suggesting that splicing factors are being relocated away from SFCs (Huang and Spector, 1996; Misteli *et al.*, 1997). Live cell imaging using GFP-tagged splicing factors has also shown that the components of SFCs are in constant flux (Kruhlak *et al.*, 2000).

Labelling of transcription foci, however, suggested that SFCs are not sites of transcription (Cmarko *et al.*, 1999), although transcribed genes and transcription foci were found in the periphery of these domains (Wei *et al.*, 1999; Xing *et al.*, 1995). Likewise, it has been suggested that splicing of pre-mRNA does not occur at these sites, but that they function as storage sites for splicing factors (Misteli, 2000). Poly-adenylated RNA has been localised to these speckles, though it has been proposed that these are defective or non-coding sequences (Huang *et al.*, 1994). Introduction of

exogenous RNA into the nucleus showed that the RNA from intron-containing genes colocalised with splicing factors that may have been released from SFCs (Huang and Spector, 1996). This has been confirmed using live-cell experiments, where GFP-tagged splicing factors were shown to leave SFCs to target sites of transcription (Misteli *et al.*, 1997). It has been also shown using RNA-FISH that transcripts for certain genes extend into SC-35 domains (Smith *et al.*, 1999; Xing *et al.*, 1995). Thus SC-35 domains, which include PFs, may be splicing sites for subsets of genes, although what distinguishes these subsets is yet to be determined.

1.2.2.4 Other nuclear bodies

Examples of less well-characterised nuclear structures are given in Table 1.3. This list is by no means complete. New intranuclear structures are being discovered all the time, and many of these have still to be characterised structurally, let alone functionally, and will not be mentioned.

1.2.2.5 Nuclear matrix

As discussed earlier, in mitosis, as the chromatin condenses, a protein scaffold is proposed to form the backbone to which SARs within the chromatin are attached. It has therefore been suggested that a similar protein structure, the nuclear matrix exists in interphase, to which chromatin and nuclear structures may be fixed. At present, no agreement as to the existence, let alone nature, of the matrix has been reached (Nickerson, 2001; Pederson, 2000).

The concept of a nuclear matrix was based on, and best defined by, the observation that certain chemical treatments, including salt extraction, resulted in a residual insoluble fraction (for review see Nickerson, 2001). Although extraction experiments were performed more than 50 years ago, it was Berezney and Coffey who formally proposed the presence of a matrix (Berezney and Coffey, 1974). The idea of a matrix has since been formulated from several observations. Salt extractions of intact nuclei have resulted in a lattice-like structure remaining within the nuclear shell, as shown by EM studies (Berezney and Coffey, 1977; Capco *et al.*, 1982). This lattice contains RNPs and proteins, and communicates with the nuclear lamina. GFP-tagged proteins isolated from the matrix likewise show this structure in live Drosophila cells (Oegema *et al.*, 1997). Sceptics, however, claim that such experiments are artefactual, or do not fit known

Table 1.3: Nuclear bodies that are less characterised (in comparison to the nucleolus, Cajal bodies, speckles and PML bodies).

Body	Appearance/behaviour	Postulated function(s)	References
GEMS	Adjacent/overlap Cajal bodies, contain SMN protein	RNP maturation, involved in spinal muscular atrophy	(Liu and Dreyfuss, 1996)
HSF1 granules	Heterochromatin of chromosome 9	Heat shock/stress, related to transcription of heat shock proteins?	(Jolly *et al.*, 2002)
Paraspeckles	Associate with nucleolus, contains PSP1	Unknown. Transcription?	(Fox *et al.*, 2002)
OPT domain	Few, small (1.5um), G1-phase. Associate with chromosomes 6 and 7	Transcription of specific genes	(Pombo *et al.*, 1998)
Perinucleolar compartment	Touching the nucleolus, contains RNA pol III and snRNAs.	Transcription/RNA processing	(Huang *et al.*, 1997)
Cleavage body	Small foci adjacent to Cajal bodies	RNA processing	(Schul *et al.*, 1999)
GATA-1 body	Small foci	Transcription	(Elefanty *et al.*, 1996)
PcG domain	Pericentromeric foci, associate with heterochromatin	Transcription repressor	(Saurin *et al.*, 1998)

GEMS – gemeni of coiled bodies. OPT – Oct1, PTF Transcription domains. HSF1 – heat shock factor-1. SMN - . PSP1 – paraspeckle protein-1. PcG – polycomb group granules.

models of molecular mobility within the nucleus (including those of the GFP-tagged proteins) (Pederson, 2000).

As mentioned, chromatin itself is proposed to be anchored to the matrix. Furthermore, DNase treatment of cells prior to matrix extraction has revealed sequences which are specifically matrix-bound, called matrix attachment regions (MARs), corresponding to SARs in mitosis (Craig *et al.*, 1997; Heng *et al.*, 2004). Intervening regions extend out into loops (Jackson *et al.*, 1990). Transcription and replication complexes are thought also to be matrix-associated (see next section), as are some nuclear structures such as PML bodies (see Section 1.3). However, rather than being static, the dynamic nature of the matrix has been noted (Berezney and Coffey, 1977). Furthermore, matrix-associated structures, ranging from transcription factors and chromatin, to PML bodies are found to be facultatively, rather than constitutively bound to it. Thus the nuclear matrix may also serve a regulatory role, rather than a passive or structural one.

1.2.3 Functional nuclear compartments

Most of the nuclear structures described so far have been discovered as a result of either their structural homogeneity, allowing visualisation by phase contrast microscopy, electron microscopy or subnuclear separation by centrifugation. In most cases, these characteristics may reflect an underlying function provided by these structures. However, such physical structures are not responsible for all nuclear activity. Several other nuclear structures do not form discrete bodies, but may still be considered as compartments that play an important role in gene regulation. They include sites of transcription, DNA replication or repair. These compartments suggest that a level of spatial and temporal organisation exists in the nucleus, in addition to chromosome territories and their loci on one hand, and macromolecular structures in the ICD on the other.

1.2.3.1 Transcription domains

Unlike the transcription of rDNA genes, which occurs specifically in the nucleolus, the transcription of mRNA by RNA pol II (as well as snRNA transcription by RNA pol III) occurs throughout the nucleoplasm. Sites of transcription have been studied using tritium- or halogen- labelled nucleotides (Jackson *et al.*, 1993). It was determined by microscopy that in HeLa cells there are about 10,000 non-nucleolar sites or 'factories', 8,000 of which were thought to be RNA pol II mediated, the remaining being

transcribed by RNA pol III (Pombo *et al.*, 1999). Biochemical studies suggest there are approximately 65,000 RNA pol II transcripts, and 10,000 RNA pol III transcripts (Jackson *et al.*, 1998; Pombo *et al.*, 1999). Thus, it has been proposed there are 8 RNA pol II, and 5 RNA pol III molecules per transcription focus or 'factory' (Martin and Pombo, 2003).

Traditionally, it was thought that transcription complexes tended to be mobile, attaching to the more immobile promoter sites of transcribed genes. However, the discovery of these transcription factories as large protein complexes consisting of several subunits has prompted the theory that these foci are immobile, and that chromatin moves along them as genes are transcribed (Cook, 1999). It as also been shown that certain transcription factors are matrix-bound (Mortillaro *et al.*, 1996; Reyes *et al.*, 1997). It has therefore been proposed that these transcription factories are located at matrix-associated sites, corresponding to DNA loop bases, which contain active genes (Gerdes *et al.*, 1994).

While immunofluorescence studies of RNA pol II showed consistent colocalisation with transcription foci, other transcription co-factors showed variable colocalisation with transcription foci (Grande *et al.*, 1997). This suggests that many different factors are normally located away from active transcription sites. For example, the GFP-tagged glucocorticoid receptor was found to shuttle rapidly between the nucleoplasm and chromatin regulatory elements upon induction (McNally *et al.*, 2000). Moreover, some matrix-bound factors do not overlap with sites of transcription (Zeng *et al.*, 1998); these specific matrix-bound factors may also be storage or assembly sites.

Although transcription foci are scattered throughout the nucleus, specific interactions with nuclear structures have been noted. These foci, as already noted, are found both at the surface and in the interior of chromosome territories (Verschure *et al.*, 1999). These sites are also present at the periphery of SFCs, Cajal bodies, PML bodies and OPT domains (see above, and Section 3.1). However, as mentioned, such associations are not absolute. Furthermore, the limited number of these structures in the nucleus (ranging from 1 to 20), compared with the more speckled pattern of transcription foci (more than 1,000), suggests that these larger structures are not transcription sites themselves.

1.2.3.2 Replication domains

As suggested by replication banding patterns, DNA replication in S-phase is temporally and spatially ordered along the chromosome. Studies have also looked at the sites of DNA replication in the interphase nucleus during S-phase by labelling nascent DNA (as for replication timing studies) in both mammalian and plant cells (Jackson and Pombo, 1998; Ma *et al.*, 1998).

Replication occurs in replication foci, which may consist of 10 to100 adjacent replicons that are simultaneously replicated (Jackson and Pombo, 1998; Ma *et al.*, 1998). Berezney and colleagues suggest that replication foci are heterogeneous in size being composed of clusters of replicons that differ in both size and number (with some foci being composed of a single large replicon) (Ma *et al.*, 1998). The DNA in each of these replication foci is thought to be replicated in less than one hour (Hand, 1978; Jackson and Pombo, 1998) .

Replication foci appear to be immobile sites, with new foci forming *de novo* (Leonhardt *et al.*, 2000). It has been suggested that they are present at bases of chromatin loops (Gerdes *et al.*, 1994). Replication machinery is also found attached to the nuclear matrix after extraction of the cells (Hozak *et al.*, 1993). Furthermore, the isolated nuclear matrix is capable of synthesising DNA at replication sites that are indistinguishable from those visualised in intact cells (Nakayasu and Berezney, 1989). It is therefore proposed that these immobile foci contain all the factors necessary for DNA replication. In addition to nascent DNA and replication factors, these foci have been shown to include proteins involved in cell cycle control (Cardoso *et al.*, 1993).

Specific spatial distributions of replication foci in different S-phase stages have been noted using microscopy (see Chapter 5 for examples) (O'Keefe *et al.*, 1992). A diffuse nuclear pattern is seen in early S-phase corresponding to gene-rich and transcriptionally active regions. In late S-phase, replication is then limited to larger foci in the nuclear and nucleolar periphery, corresponding to heterochromatic regions. Further details of the replication patterns and their relationship to other nuclear structures will be discussed in Chapter 5.

1.2.3.3 DNA repair sites

DNA damage, if unrepaired, leads either to cell death or genomic instability, and confers a predisposition to cancer formation. Eukaryotic cells have a variety of repair mechanisms which sense and repair DNA, including nucleotide excision repair (NER), homologous recombination or non-homologous end-joining (NHEJ) (for reviews see Bubulya and Spector, 2004; Stein *et al.*, 2003). In these processes, proteins involved in transcription and DNA replication, including CBP complexes, SWI/SNF, PCNA and replication protein A (RPA) are often recruited (reviewed in Gontijo *et al.*, 2003; Stein *et al.*, 2003). As with transcription and DNA replication, DNA repair is recognised to be coordinated spatially and temporally in the nucleus. For example, the DNA-protein kinase complex is active during G1-phase, the BRCA1/RAD51 complex during late S/G2-phase, and the Mre11/RAD50/NBS1 complex throughout the cell cycle (Stein *et al.*, 2003).

Unlike transcription and replication, visible DNA repair sites can often only be detected following treatment of cells with DNA damaging agents. Using UV-induced DNA damage, the formation of double strand breaks (DSB) is visualised either by focussed irradiation of a cell, or by labelling single-stranded DNA using BrdU in non-denatured chromatin. Using confocal microscopy, it was found that DNA repair complexes and proteins relocate to sites of DNA damage. One example of these proteins is TFIIH, a subunit of the general transcription machinery also involved in NER. Following DNA damage, 40% of GFP-tagged TFIIH was shown to be recruited to these sites within 2 minutes, and to remain for about 4 minutes. It was suggested that TFIIH shuttles between transcription and repair sites by stochastic mechanisms (Hoogstraten *et al.*, 2002). Similarly, the phosphorylated histone protein H2AX rapidly accumulates at DSB sites (Paull *et al.*, 2000). H2AX then recruits other repair proteins to these sites, including the BRCA1/RAD51 and Mre11/RAD50/NBS1 complexes. In contrast, larger structures containing these complexes, as well as PML protein (see Section 1.3.7.7 and Tashiro *et al.*, 2000) appear to form over several hours. Since DNA repair is usually repaired within 90 minutes, such late bodies may reflect unrepairable lesions (Petrini and Stracker, 2003).

To summarise, a diagrammatic representation of the different nuclear structures is given in Figure 1.2. As seen, the biochemical processes involved in the nucleus, principally those of transcription and post-transcriptional processing, DNA replication and DNA

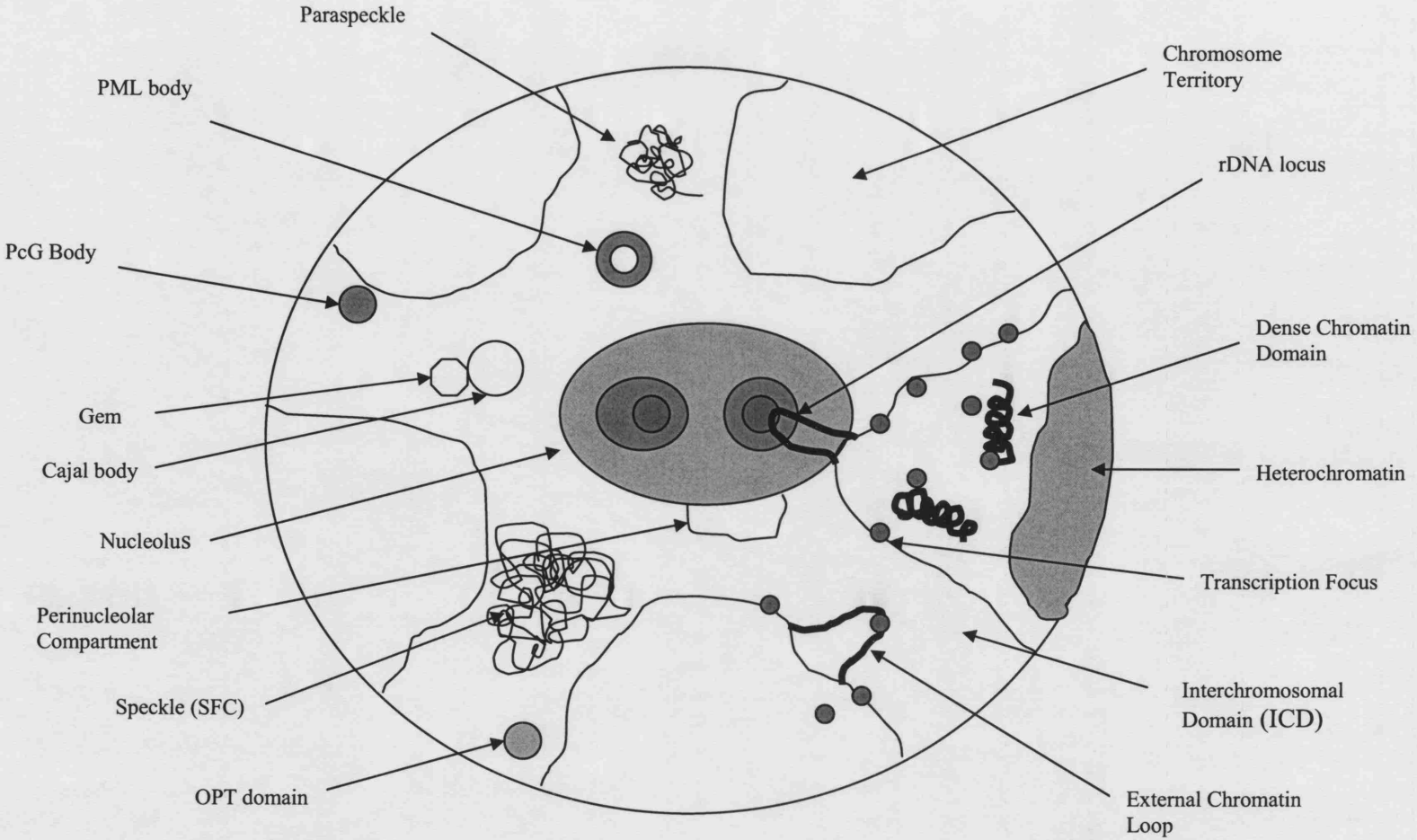

Figure 1.2. Diagram of nuclear organisation.
The commonly described nuclear structures are shown.

repair, are structurally and temporally organised and associated with the so-called 'structural' nuclear domains. This section has not dealt with DNA repair or other nuclear processes, as they are generally not studied in the context of nuclear organisation. These processes will be discussed in the appropriate sections later.

1.3 PML nuclear bodies

Where, then, does the PML nuclear body fit in the functional organisation of the nucleus? As mentioned, PML bodies have been intensively researched due to their clinical significance. However, unlike many of the other nuclear bodies or structures, a predominant role has not been found for PML nuclear bodies. Rather, many diverse nuclear roles have been linked with these bodies. An in-depth review of their biology is therefore important in understanding the work done in this thesis.

1.3.1 Discovery of PML nuclear bodies

PML bodies were initially recognised using the electron microscope in the 1960s as 'dense granular bodies and pale round structures, at times showing a complex lamellar pattern' (for review see Maul, 1998). Autoimmune sera from patients with primary biliary cirrhosis, such as those directed against the Sp100 protein, were subsequently found to label these nuclear bodies, thus relating them to clinical disease (Szostecki *et al.*, 1990).

Interest in PML bodies burgeoned when they were linked to a form of leukaemia, acute promyelocytic leukaemia (APL). In the early 1990s, the t(15;17) chromosome translocation, found specifically in APL, was shown to give rise to a fusion between the genes for the so-called promyelocytic leukaemia (PML), and the retinoic acid receptor-alpha (RARα) proteins (de The *et al.*, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991). The fusion protein, PML-RARα, causes a block in the differentiation of promyelocytes in the bone marrow, and is sufficient for the development of leukaemia (see Section1.3.8). Subsequently, it was found that the PML protein is a major constituent of the nuclear bodies mentioned above, which is found in a variety of normal cells (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). In acute promyelocytic leukaemia, the PML-RARα fusion protein acts in a dominant negative manner, leading to the redistribution of PML nuclear bodies into microspeckles. As a result of the complex history of their discovery, PML bodies have been variously termed nuclear dots 10 (ND10), PML (nuclear) bodies/NBs, PML oncogenic domains

(PODs) or Kremer (Kr) bodies. In the remainder of this thesis, the term PML bodies will be mainly used.

1.3.2 Structure of the PML protein

The *PML* gene at chromosome band 15q22 is 35 kb in length and contains nine exons (Fagioli *et al.*, 1992). It is flanked by two CpG islands, which are separated by a variable distance, due to large insertion/deletion polymorphism at the 3' end of the gene (Goy *et al.*, 1995).

Several alternative splice variants of the PML protein have been noted, principally involving the C-terminal exons (Fagioli *et al.*, 1992) (see Figure 1.3). Most cell lines studied, however, express a similar set of isoforms. Recently, these isoforms have been integrated into a new classification scheme, and named PML-I to -VII (Jensen *et al.*, 2001). All isoforms have a characteristic region encoded by exons 1 to 3. This consists of:

1. A cysteine-rich zinc finger, termed a RING (Really Interesting New Gene) finger (Borden *et al.*, 1995),
2. Two further cysteine-rich zinc fingers, termed B-boxes (Borden *et al.*, 1996),
3. An alpha-helical coiled-coil region with eight heptad repeats with hydrophobic amino acids in the first and fourth positions (Kastner *et al.*, 1992).

Together, these domains (amino acid sequences 57aa to 360aa) form the RBCC motif, also known as the tripartite motif (TRIM) (Reymond *et al.*, 2001). Mutations directed against the different parts of this motif have shown that the whole structure is required for the homodimerisation of the PML protein, interactions with other proteins and subsequent formation of the PML body (Borden *et al.*, 1995; Kastner *et al.*, 1992). In addition, the different domains have different functions. The RING finger was initially thought to facilitate binding to DNA (Fagioli *et al.*, 1992; Goddard *et al.*, 1991), but it was subsequently found that the positively charged structure precluded DNA binding (Borden *et al.*, 1995; Saurin *et al.*, 1996). These studies show that while the B-boxes are responsible for PML body formation, they are not involved in homodimerisation of the PML proteins. Finally, the coiled-coil domain is involved in multidimerisation of PML protein and also heterodimerisation to the PML-RARα fusion product and other proteins (Kastner *et al.*, 1992; Le *et al.*, 1996).

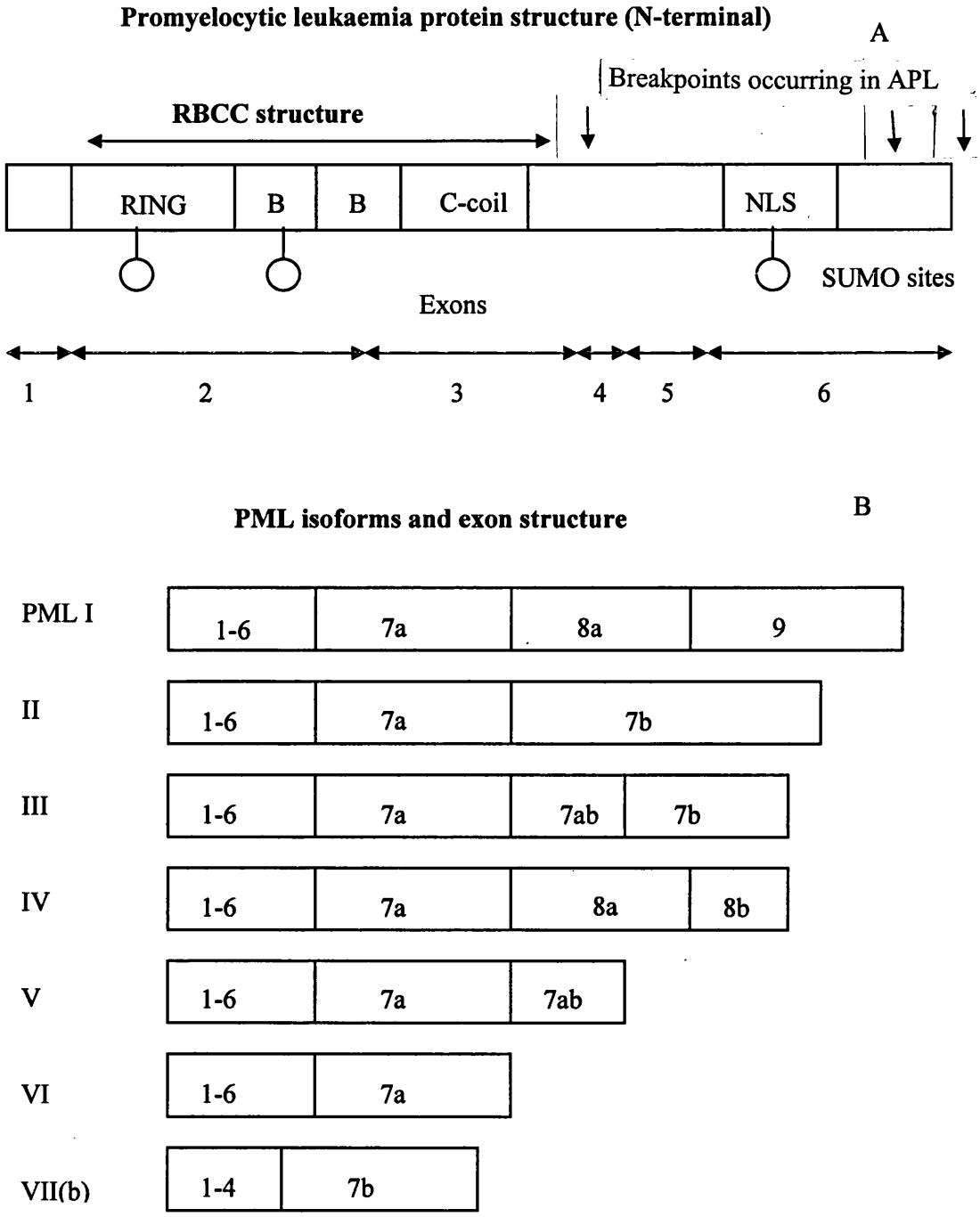

Figure 1.3. The structural organisation of the PML protein.
A: Diagram of the common N-terminal end of the PML protein, with the RBCC/TRIM motif. B: The common splice variants/isoforms of the PML protein, with variations in the C-terminal end.

Further along the gene (coding for amino acid sequences 394aa to 552aa), exons 4, 5 and 6 are subject to alternative splicing (Fagioli *et al.*, 1992; Jensen *et al.*, 2001). Exon 6 contains a nuclear localisation signal (NLS) (Le *et al.*, 1996). Splice variants without this NLS have a cytoplasmic distribution. Finally, the C-terminus contains an acidic serine/proline-rich domain, which is highly variable in length due to alternative splicing of exons 7-9 (Fagioli *et al.*, 1992). This region is rich in potential phosphorylation sites but its biological function is unknown (Kastner *et al.*, 1992; Pandolfi *et al.*, 1991).

The presence of the different isoforms results in up to 20 protein bands when analysed on a Western blot (Fagioli *et al.*, 1992). These range in size from 50kDa to more than 100kDa. As mentioned, some isoforms have a cytoplasmic distribution, although the majority show a predominantly nuclear pattern (Jensen *et al.*, 2001). Within the nucleus, the PML protein is found largely in PML bodies which are nuclear matrix bound (Stuurman *et al.*, 1992), although some protein is present in a soluble form in the nucleoplasm (Chang *et al.*, 1995).

1.3.2.1 The RBCC/TRIM protein family

Proteins containing the RING finger motif is involved in diverse functions, including DNA repair, transcription and protein degradation via E3 ligase activity (Saurin *et al.*, 1996, Joazeiro and Weissman, 2000). The RBCC motif is present in at least 37 protein families, many novel with unknown functions (Reymond *et al.*, 2001). In these proteins, the order of the RBCC domains is conserved, although the number of B-boxes may vary. As with PML, members of the RBCC/TRIM protein family can homodimerise. For example, another RBCC family protein, transcription intermediary factor-1b (TIF1b), homodimerises to form a nuclear compartment distinct from PML, the KRAB (Kruppel-associated box) transcriptional repressor domain (Peng *et al.*, 2002). This effect is specific to the RBCC domain of TIF1b, as substitutions with RBCC domains from other proteins fail to reproduce this function. Another RBCC protein, the Ret Finger Protein (RFP) heterodimerises with PML in PML bodies (Cao *et al.*, 1998). Thus, despite being a family of proteins with different functions, it is thought that the RBCC/TRIM motif acts to organise subcellular domains (Reymond *et al.*, 2001).

1.3.3 Control of PML expression

The PML gene promoter contains an interferon-stimulated response element (ISRE) as well as a gamma-activation sequence (GAS) (see Section 4.1.3 for a description of the

interferon pathway). These allow binding of signal transducer and activator of transcription (STAT) proteins in response to both type I (alpha and beta) and type II (gamma) interferons (IFNs) (Stadler *et al.*, 1995). More recently, a p53-binding site has also been found in the promoter region of the PML gene (de Stanchina *et al.*, 2004). This has important implications for the role of PML in cell cycle control (see Section 1.3.7).

Interferons are immuno-modulatory cytokines. In particular, IFNα and -β are potent anti-viral mediators. Treatment with IFNα, -β and -γ result in upregulation of PML (both protein levels and number of PML bodies) in a variety of cell lines, as a primary response (without an intermediary protein synthesis) (Lavau *et al.*, 1995). Other cytokines tested in this study, including interleukin-1 (IL-1), IL-6, Tumour Necrosis Factor-alpha (TNFα) and thromboxane B2 had no effect on PML expression. Another study also showed that IL-2 and PHA did not affect the appearance of PML bodies, despite stimulating resting lymphocytes (Gambacorta *et al.*, 1996). However, induction of differentiation of haematopoietic stem cells by IL-3, granulocyte/monocyte-colony stimulating factor (GM-CSF) and erythropoietin, is associated with upregulation of PML expression (by mRNA and protein quantification), although it is not known if this effect is direct (Labbaye *et al.*, 1999). In rat uterine epithelium, the number of PML bodies is also increased by oestrogens (Padykula *et al.*, 1981).

Post-transcriptional regulation of PML protein expression (and thus PML bodies) has also been suggested. Chan *et al*, for example, found that PML protein levels, but not mRNA levels were increased by treatment of cells by ionizing radiation (Chan *et al.*, 1997). One modification of the PML protein that is functionally important is SUMOylation. Following translation, the PML protein is modified by the ubiquitin-like protein, termed small ubiquitin-like modifier-1 (SUMO-1) (Muller *et al.*, 1998). Although SUMO-1 is similar to ubiquitin, modification of proteins by SUMO-1 does not mark them for degradation as in the ubiquitination pathway. The 11.5 kDa SUMO-1 protein covalently binds to lysine residues at three sites: in the RING finger, in the B1-box and in the NLS (Kamitani *et al.*, 1998). SUMO modification has been shown to be important for the localisation of PML proteins into PML bodies (Muller *et al.*, 1998; Zhong *et al.*, 2000a). Mutations of the lysine residues in the PML protein leads to a failure of PML body formation.

1.3.4 The pattern of PML bodies in different tissues

PML expression has been examined by microscopy in a variety of cell lines and tissues. Although the PML protein is expressed in all cell lines tested, the number of PML bodies varies, with cell lines HeLa and HepG2 having notably fewer PML bodies than Hs27 fibroblasts or peripheral lymphocytes (Koken *et al.*, 1995). As mentioned, APL cells, including the NB4 cell line, show a microspeckled pattern with numerous dots (Daniel *et al.*, 1993).

Human tissue sections with different cell types and disease states have also shown marked variability in the number of PML bodies. (Gambacorta *et al.*, 1996; Koken *et al.*, 1995; Terris *et al.*, 1995). It must be noted, however, that due to different fixation methods (liquid nitrogen frozen or paraffin embedded), extraction processes and antibody selection, direct comparisons between studies remains difficult. For example, it has been suggested that the PML protein can only be detected in paraffin-embedded tissues when it is overexpressed (Gambacorta *et al.*, 1996). Furthermore, it is often not possible to detect discrete PML bodies in tissue samples. Rather, the whole nucleus often stains for reactivity to anti-PML antibodies.

'Normal' biopsy specimens were analysed from liver, thymus, tonsils, skin and kidneys (Gambacorta *et al.*, 1996; Koken *et al.*, 1995). It was found that vascular endothelium, macrophages, fibroblasts, adipocytes, myocytes, Kuppfer cell, glial cells and large neurons show strong immunoreactivity to PML, while hepatocytes, kidney glomerular cells and colon glandular cells show weak or negative staining. Basal keratinocytes showed moderate to strong PML expression, but this is lost in the superficial layers of epithelia. Another study suggested that PML expression is increased in mature and differentiated cell types, including those mentioned above (Flenghi *et al.*, 1995). Sex-hormone dependent cells, such as breast ductal epithelium, endometrium and testicular Leydig cells have increased PML body sizes and numbers, compared with other cell types (Cho *et al.*, 1998b). PML is, however, absent in male and female germ cells (Cho *et al.*, 1998b; Gambacorta *et al.*, 1996). Furthermore, there is a correlation between the hormone level and the number of PML bodies (Koken *et al.*, 1995). For example, the number of PML bodies in the endometrium varies with serum oestrogen levels. PML bodies are also detected in myeloid precursors in the bone marrow, but less expressed in maturing and circulating monocytes and granulocytes (Daniel *et al.*, 1993; Labbaye *et al.*, 1999; Nason-Burchenal *et al.*, 1996). However, in circulating blood cells, PML

bodies and protein levels are upregulated by IFNγ (Nason-Burchenal *et al.*, 1996). In lymphoid cells, PML bodies are expressed mainly in mature T and B cells, but not in the germinal centre of spleen, lymph nodes and Peyer's patches, or in circulating lymphocytes (Daniel *et al.*, 1993; Flenghi *et al.*, 1995; Lam *et al.*, 1995; Nason-Burchenal *et al.*, 1996).

PML expression is also altered in many pathological tissues. As described above and in the previous section, there is an increase in the number and size of PML bodies following interferon-induction. Such cytokine activation is likely to explain the observation that PML is highly expressed in inflamed tissues, such as psoriasis and hepatitis, inflammatory cells around cancers, and activated fibroblasts (Aractingi *et al.*, 1997; Gambacorta *et al.*, 1996; Koken *et al.*, 1995; Terris *et al.*, 1995). The relationship between PML expression and sex hormones is also shown in fibroadenomas of the breast, where a correlation between oestrogen and progesterone receptor status and the number and sizes of PML bodies is seen (Cho *et al.*, 1998b). With regard to tissues responsive to or inducible by hormones and cytokines, however, the effects of such induction often are wide-ranging, and rates of cellular proliferation and protein synthesis may be increased. It has been suggested that the presence or upregulation of PML bodies is secondary to transcriptional activity or cell cycling (Lam *et al.*, 1995; Terris *et al.*, 1995). This may also be the basis for the varied patterns and expression of PML bodies among different cancers (see Section 1.3.8). Taken together, PML expression in different cell types may be a function of cellular proliferation, differentiation or activity.

1.3.4.1 The structure and distribution of PML bodies in the nucleus

The sizes of PML bodies vary from 0.2 to 1 μm in diameter (Maul *et al.*, 1995). They have been described as doughnut shaped, but a more accurate three-dimensional term would be a hollow sphere, as detected using 3D confocal microscopy. Subsequently, electron spectroscopic imaging has revealed both a protein shell and core, with the centre of the core sometimes showing a depletion of mass (Boisvert *et al.*, 2000). Phosphorus mapping shows that there is no nucleic acid within the core, but nascent RNA is found in the periphery of these bodies.

On average, there are between 5 and 30 PML bodies per nucleus in cell lines studied (Koken *et al.*, 1995). The number and sizes of PML bodies was reported to change

during the cell cycle. In proliferating cells, upon exit from mitosis, there is a gradual increase in number of PML bodies throughout G1-phase. In early G1, some PML protein may be found outside the nucleus in cytoplasmic bodies (Everett *et al.*, 1999b; Koken *et al.*, 1995). At S-phase, the number of PML bodies increases to a mean of more than 20, and a dispersed nuclear pattern is also seen (Terris *et al.*, 1995). The number of PML bodies decreases again during G2 until only 2 to3 large perichromosomal dots are seen in mitosis (Koken *et al.*, 1995). This cell-cycle pattern has however been disputed. For example, using HeLa cells, it was found that the number of PML bodies reaches a peak in G1, compared with the later phases (Chang *et al.*, 1995). More recently, no significant change in the number of PML bodies was noted in cycling SK-N-SH and 293 cells (Eskiw and Bazett-Jones, 2002). It may be that the phenomenon is cell type specific. In addition, various reports have suggested that fibroblast cells which are arrested in G0 can have either fewer PML bodies (Terris *et al.*, 1995), or more and larger PML bodies (Maul *et al.*, 2000). Senescence also appears to upregulate the expression of PML protein and PML bodies, which may be related to Ras expression (see Section 1.3.8).

The PML protein is found to be nuclear matrix bound (Stuurman *et al.*, 1992), and the PML bodies lie in the interchromosomal domain (Plehn-Dujowich *et al.*, 2000). Spatial associations have also been found between PML bodies and other nuclear structures. These will be discussed in Chapter 3.

Various stresses have also been shown to affect the distribution of PML bodies. Heat shock and exposure to cadmium ions, as well as HSV-1 viral infections result in the redistribution of PML bodies into a microspeckled pattern throughout the nucleus (Maul *et al.*, 1995). This dispersal occurs rapidly without new protein synthesis, and has subsequently been suggested to be due to SUMO release, and fission or budding of pre-existing PML bodies (Eskiw *et al.*, 2003). In addition, increased numbers of PML bodies are also seen in response to DNA damage by cisplatin and γ-irradiation, without an increase in PML mRNA levels (Chan *et al.*, 1997). Rather than being a general dispersal of PML bodies, specific alterations of the composition of PML bodies have been shown, with heat shock resulting in release of Daxx and Sp100 from PML bodies, while cadmium ions cause the PML protein, but not Sp100 to be released from these bodies (Nefkens *et al.*, 2003). It remains to be seen whether this drastic change has a purpose, or if it is simply a consequence of cellular stress.

There is increasing use of live-cell imaging of PML bodies by means of GFP-tagged PML or Sp100 transfection. An early paper showed that unlike Cajal bodies, they are relatively immobile (Muratani *et al.*, 2002). Using Sp100-tagged proteins, more than 25% of PML bodies detected in this way were found to be static. A further 63% show limited oscillation-type movements. Only 12% are highly mobile, moving at rates of 4 – 7 μm/s, which are ATP-dependent and dependent on myosin. Variations are also seen in different cell types, with HeLa cells showing no moving PML bodies, while fibroblasts show more mobility. Another study however showed that Sp100- and PML-GFP proteins have different behaviours, with highly mobile smaller bodies containing Sp100 but not PML shuttling between larger PML-containing bodies (Wiesmeijer *et al.*, 2002). More recently, changes in shape and movements have been seen in response to cellular stress (see above) (Eskiw *et al.*, 2003). The mobility of PML bodies is also related to attachment of chromatin, and transcriptional inhibitors resulted in retraction of chromatin from PML bodies, coinciding with the formation of new PML bodies by fission (Eskiw *et al.*, 2004).

1.3.5 Other proteins in PML bodies

The PML protein is the main structural component of PML bodies. Following SUMO modification, the PML protein aggregates to form PML bodies. This step is essential for the subsequent recruitment of other proteins into PML bodies, as loss of the PML protein or mutations of the *PML* gene prevents the incorporation of proteins such as BLM, Daxx, cAMP Response Element Binding (CREB)-binding protein (CBP), SUMO-1 and Sp100. This results in an aberrant distribution of these proteins (Ishov *et al.*, 1999; Zhong *et al.*, 1999b; Zhong *et al.*, 2000a). The proteins that have been detected in PML bodies form a diverse and (at present) expanding list (see Table 1.4). These proteins have implicated PML bodies in gene transcription, repair, cell cycle control and apoptosis (see Section 1.3.7).

It must be noted that the conditions under which the association of different proteins with PML bodies is assayed is not uniform. Some associations are physiological and endogenous, while others are conditional, or only occur in the event of overexpression or inactivation. For example, the tumour suppressor gene, BRCA1, colocalises with these bodies only when overexpressed (Maul *et al.*, 1998). The retinoblastoma protein is found in PML bodies, but only in the unphosphorylated, inactive form (Alcalay *et al.*, 1998). The methods used to examine the association of proteins with PML bodies (or

Table 1.4: Non-viral protein components of PML protein/bodies.

Protein	Proposed function	Characteristics	References
PML	Structural	Endogenous	-
Sp100	Structural/transcription	Endogenous, IFN	(Sternsdorf *et al.*, 1997)
SUMO-1	Structural, regulator	Endogenous	(Boddy *et al.*, 1996)
SENP-1	Structural via SUMO		(Nefkens *et al.*, 2003)
Ubc9	Structural, regulator		(Gong *et al.*, 1997)
HAUSP	Ubiquitin family	Associates with viral proteins	(Everett *et al.*, 1997)
NDP 52,53,55,65	Unknown	Endogenous	(Ascoli and Maul, 1991) (Korioth *et al.*, 1995)
HP1	Chromosome modelling	Interacts with sp100, cell cycle	(Seeler *et al.*, 1998)
HMG1/2	Chromosome modelling	Sp100 partner/variant	(Seeler *et al.*, 2001)
Proteasome	Protein degradation	IFN upregulated (immunoproteasome)	(Fabunmi *et al.*, 2001)
TIF1a	Transcription	Acts with PML on RARα/RXR	(Zhong *et al.*, 1999a)
CBP	Transcription	Antibody dependent Subset of cells	(LaMorte *et al.*, 1998)
EIF4E	MRNA transport		(Cohen *et al.*, 2001)
Sp1	Transcription	Immunoprecipitation (IP) only	(Vallian *et al.*, 1998a)
Daxx	Transcription, apoptosis	Endogenous	(Everett *et al.*, 1999a)
DNAHelII	Transcription	IFN upregulated	(Fuchsova *et al.*, 2002)
RNApolII	Transcription	Hypophosphorylated form	(von Mikecz *et al.*, 2000)
Nur77	Nuclear receptor, transcription	Endogenous, overexpressed	(Wu *et al.*, 2002b)
AP-1	Transcription, growth	Overexpressed (IP only)	(Vallian *et al.*, 1998b)
β-catenin	Transcription	Overexpressed	(Shtutman *et al.*, 2002)
GRIP1	Nuclear receptor, transcription	Overexpressed	(Baumann *et al.*, 2001)
HDAC	Transcription	PML IV	(Wu *et al.*, 2001)
PRH	Myeloid differentiation, transcription	In APL/myeloid cells	(Topcu *et al.*, 1999)
GAPDH	RNA metabolism	RNA dependent	(Carlile *et al.*, 1998)
P proteins	Translation	Associate with viral proteins	(Borden *et al.*, 1998)
Int-6/eIF3	Translation		(Desbois *et al.*, 1996)
pRb	growth control/ transcription	Inactive form, Ras, interacts with PML IV	(Alcalay *et al.*, 1998)

p53	growth control/ tumour suppressor	overexpressed, repair sites, interacts with PML IV	(Guo *et al.*, 2000)
MDM2	growth control/ tumour suppressor	IFN	(Wei *et al.*, 2003)
Chk2	Checkpoint / apoptosis	Dissociates from PML with UV	(Yang *et al.*, 2002)
BLM	DNA repair	Cell cycle dependent	(Zhong *et al.*, 1999b)
TRF1/2	Telomere maintenance	ALT cells	(Yeager *et al.*, 1999)
NBS1 (p95)	DNA repair, telomere	ALT cells, repair sites	(Wu *et al.*, 2000) (Carbone *et al.*, 2002)
Mre11	DNA repair, telomere	ALT cells, repair sites	(Wu *et al.*, 2003a) (Carbone *et al.*, 2002)
RAD51	DNA repair, telomere	ALT cells, repair sites	(Wu *et al.*, 2003a)
RAD51/52	DNA repair, telomere	ALT cells, repair sites	(Yeager *et al.*, 1999) (Bischof *et al.*, 2001)
RP-A	DNA repair, telomere, replication	ALT cells, repair sites	(Yeager *et al.*, 1999) (Bischof *et al.*, 2001)
WRN	DNA repair, telomere	ALT cells, repair sites	(Blander *et al.*, 2002)
Bach2	Transcription	Oxidative stress	(Muto *et al.*, 2002)
Topors	DNA repair/ replication	Dissociates with DRB treatment	(Rasheed *et al.*, 2002)
HSF2	Heat shock protein	overexpressed	(Goodson *et al.*, 2001)
Tax	Viral replication, oncogene	overexpressed	(Doucas and Evans, 1999)
ISG20	Anti-viral response	overexpressed, IFN upregulated	(Gongora *et al.*, 1997)
HIPK2	Growth control, transcription	overexpressed	(Moller *et al.*, 2003)
BRCA1	Tumour suppressor	overexpressed, ALT cells	(Maul *et al.*, 1998) (Wu *et al.*, 2003a)
HGCNP	Unknown	overexpressed, adjacent	(Maul, 1998)
RFP	Myeloid differentiation	Subset of PML bodies, in APL	(Cao *et al.*, 1998)
PLZF	Myeloid differentiation	In APL cells, adjacent to PML	(Ruthardt *et al.*, 1998)
Sp110	Transcription	Myeloid cells, related to Sp100, IFN	(Bloch *et al.*, 2000)
Sp140	Myeloid differentiation	Myeloid/APL cells, related to Sp100, IFN	(Bloch *et al.*, 1996)
Ataxins	Polyglutamine mutants	In mutant cells (abnormal NBs)	(Takahashi *et al.*, 2003)

with PML protein), vary from colocalisation with microscopy, co-immunoprecipitation (IP), Glutathione S-transferase (GST)-pull down or Yeast Two-Hybrid (Y2H) systems (reviewed by Seeler and Dejean, 1999). The association of CBP with PML bodies is seen only with specific antibodies, and in certain cell lines (Doucas *et al.*, 1999; LaMorte *et al.*, 1998). These issues make comparisons between studies difficult.

1.3.6 Phylogeny of the PML body/protein

Phylogenetic issues are important when considering possible functions of proteins. Our understanding of the phylogeny of the PML protein at present is limited by incomplete sequence data for the genomes of many organisms. However, PML appears to have arisen relatively late in evolution. It is found, together with PML bodies, in mice as well as humans, but not in earlier organisms studied. In fact, the RBCC/TRIM family of proteins in general appears late in evolution, not being identified in the lower organisms such as *Schizosaccharomyces cerevisiae, S. Pombe* or *Arabidopsis thaliani* (Borden, 2002). Higher eukaryotes, such as *Caenorhabditis elegans, Xenopus laevis,* newt and chicken proteins contain the RBCC motif, but with only one B-box. In *Drosophilia melanogaster,* proteins with the RBCC motif containing two B-boxes are found. However, none of the RBCC proteins from these species show sequence conservation with the mouse PML protein. In contrast, the RING finger motif alone is found in proteins in diverse organisms from *S. cerevisiae, S. pombe and A. thaliana* to man. A RING-finger protein in A thaliani, COP1, also forms nuclear bodies (Reyes, 2001), but it is not considered a precursor of PML, and humans have COP1 which forms discrete bodies distinct from PML bodies (Wang *et al.*, 1999).

As with the PML protein, PML bodies are not found in *Xenopus laevis.* This is especially important as other nuclear structures, such as Cajal bodies are found in *X. laevis* (Gall *et al.*, 1995; Gurdon and Brown, 1965). However, the eukaryotic initiation factor protein, eIF4E (a component of PML bodies in human cells) is present in nuclear bodies without PML in several species, including *D. melanogaster* and *X. laevis* (Cohen *et al.*, 2001; Lang *et al.*, 1994). Yet, in several human cell lines, the activity of eIF4E is modulated by the PML protein in PML bodies. EIF4E plays a central role in nucleocytoplasmic transport of certain mRNAs, such as Cyclin D1 mRNA. Transfection of wild-type PML into PML -/- cells results in recruitment of PML into a set of nuclear bodies containing eIF4E (Cohen *et al.*, 2001). This suggests that precursors of PML bodies may exist in earlier organisms. However, the evolution of the

PML protein may subsequently alter the associated protein pool in these bodies, and hence their underlying functions.

1.3.7 Proposed functions of the PML body/proteins

Several clues have emerged from the discussion so far regarding the role of PML bodies, primarily from their behaviour, associated proteins and phylogeny. Numerous studies have therefore examined the proposed functions of PML bodies. These cover essentially most, if not all, of the processes that occur in the nucleus. These include transcription and post-transcriptional processing, cell cycle control and growth regulation, immortalisation of cells by telomere maintenance, apoptosis and DNA replication and repair. It must be remembered however, that as with experiments involving proteins associated with PML bodies, many studies are subject to interpretation and artefacts.

1.3.7.1 PML and transcription

Of the different roles PML has been implicated in, arguably the largest body of work has been directed at a transcriptional role. Although the RING structure of the PML protein prevents binding to DNA, as mentioned earlier (Saurin *et al.*, 1996), the RING domain of PML without the coiled coil component showed a transactivating property using a GAL4-PML fusion construct (Ahn *et al.*, 1998). On the other hand, similar constructs with full-length RBCC domains were able to repress transcription, which was attributed to the coiled coil domain (Vallian *et al.*, 1997). The same group also showed that this GAL4-PML fusion protein represses transcription by interacting with histone deacetylases (Wu *et al.*, 2001). However, such fusion products are essentially artificial. The fusion product in APL, PML-RARα, has transcriptional repression properties in retinoic acid mediated transcription, but this property is attributed to alterations in the RARα protein.

Similarly, earlier studies suggested that PML bodies were involved in transcription. Earlier experiments have already shown that the number of PML bodies in different tissues in mice correlated to protein synthesis, rather than proliferation indices (Lam *et al.*, 1995). It was subsequently reported that the core of PML bodies contained RNA and RNA polymerase II (in the hypophosphorylated initiation form, RNA pol IIa) (LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000). Another study, however, showed that nascent RNA was found around, but not within PML bodies (Boisvert *et al.*, 2000). This

study also described a subjective association with nuclear regions labelled for acetylated histone proteins. The spatial association of PML bodies with transcription sites or foci has also been quantified, with 30% of PML bodies being in contact with or adjacent to transcription foci, labelled with fluorouridine (Kiesslich *et al.*, 2002). This association was increased to 70-80% in cells specifically in G1 or stimulated by IFNγ.

However, PML bodies cannot be vital for all transcriptional activity, as PML -/- cells and animals remain viable. PML protein/bodies have therefore been linked to a rapidly increasing list of specific transcription pathways:

1. The role of PML-RARα in affecting retinoic acid mediated transcription has been mentioned. In addition, PML protein acts as a cofactor for the RARα-RXR complex, and mediates its transcriptional activity on genes such as *RARB2* and *p21WAF1/CIP1* (Wang *et al.*, 1998a; Zhong *et al.*, 1999a). PML protein also interacts in this role with the TIF1 transcription factor. The transcriptional role in this pathway will be discussed later in Section 1.3.8.
2. PML was found to interact with the transcription factor Sp1, which binds to the general transcriptional machinery. The interaction was shown both *in vivo* via IP studies and *in vitro* via GST-pulldown, and interfered with the ability of Sp1 to bind DNA by mobility shift assays. Coexpression of PML with SP1 also repressed the transcription of *EGFR*, a target gene of SP1 (Vallian *et al.*, 1998a). Thus, PML is proposed to sequester SP1 and inhibit its effect.
3. The same group above also discovered, using cotransfection studies, that PML protein interacted with the AP1(fos/jun) complex, and cooperated in fos-mediated transcriptional activity at the AP1 promoter site (Vallian *et al.*, 1998b). In addition, a direct interaction between PML (via its RING domain) and fos at the AP-1 complex was found by immunoprecipitation and mobility shift assays. Both these studies (point 2, 3) did not use microscopy, thus it was not possible to determine if the interactions described occur in PML bodies.
4. PML has been shown by overexpression studies to increase the transcriptional activity of a variety of steroid receptors including the progesterone (PR), mineralocorticoid, glucocorticoid (GR) and androgen receptors (AR), although in contradiction with point 1 above, not RARα (Guiochon-Mantel *et al.*, 1995). In the case of the PR, overexpression of PML also resulted in recruitment of PR into PML bodies, mediated by the coiled coil domain of PML. However, Grande *et al* showed no systematic association between GR and PML bodies (Grande *et*

al., 1996). An orphan steroid receptor, Nur77, was also shown by similar experiments to be recruited to, but repressed by, PML bodies (Wu *et al.*, 2002b). In addition, cofactors for various nuclear receptors were also found in PML bodies, including (overexpressed) glucocorticoid receptor interacting protein-1 (GRIP-1) (Baumann *et al.*, 2001) and Tax (Doucas and Evans, 1999). Tax is an oncoprotein that represses GR and retinoid X receptor (RXR) mediated transcription. Recruitment of Tax into PML bodies by cotransfection with PML resulted in reversal of this repression. SRC-1 is a cofactor of AR that likewise is found in PML bodies when transfected (Rivera *et al.*, 2003). In this study, AR are not found in PML bodies, but activated AR causes SRC-1 to dissociate from PML bodies into a filamentous structure. Finally, the stimulation of nuclear receptor transcription is linked with the recruitment of CBP (see below) and TIF1a into PML bodies (Doucas *et al.*, 1999; Zhong *et al.*, 1999a).

5. PML has been shown to mediate the TNFα pathway. Specifically, PML protein and bodies, when overexpressed, interact with the protein, RelA/p65 (via IP and confocal microscopy), and prevents it from binding to the TNFα mediator, nuclear factor-kappa-B (NF-kB) (Wu *et al.*, 2003b). Since binding of RelA/p65 is required for the enhancer effect of NF-kB, overexpression of PML was found to repress the expression of a target gene of NF-kB, the *A20* gene (Wu *et al.*, 2002a). Tax, an oncoprotein mentioned in point 4 also mediates the effects of TNFα by interacting with NF-kB (Munoz and Israel, 1995).
6. PML bodies are also implicated in the transcriptional effects of interferon or interferon-related genes. This will be discussed fully in Chapter 4.
7. PML bodies have been linked to the Wnt signalling pathway, which is mediated by β-catenin, with LEF1/TCF1 as a cofactor. Transfection of PML facilitates the transcriptional activity of β-catenin on some, but not all target genes (for example, *ARF*, but not *cyclin D1*) (Shtutman *et al.*, 2002).
8. PML bodies colocalise with the pro-apoptotic factor and transcriptional repressor Daxx under physiological conditions, as well as by Y2H experiments (Everett *et al.*, 1999a; Ishov *et al.*, 1999). This recruitment requires SUMO-1 (Li *et al.*, 2000; Zhong *et al.*, 2000a). In the absence of PML, Daxx aggregates at condensed chromatin. Daxx represses the transcription of PAX3, itself a transcription factor, and this effect is reversed by the sequestering of Daxx into PML bodies, in co-transfection experiments (Lehembre *et al.*, 2001). In addition, Daxx opposes the transcriptional activity of GR (Lin *et al.*, 2003b). The

repression mechanism is thought to involve HIPK2 (homeobox-interacting protein kinase) (Ecsedy *et al.*, 2003; Engelhardt *et al.*, 2003; Hofmann *et al.*, 2003), which also removes Daxx from PML bodies.

9. The role of PML in transcription of pRb and p53 will be discussed below in Section 1.3.7.4, as their transcriptional activity is related to their function as growth and tumour suppressors.

With such diverse roles in transcription, one possible function of PML bodies is to coordinate the different pathways. It has been suggested that CBP is an integrator of different pathways (Chan and La Thangue, 2001; Vo and Goodman, 2001), including the nuclear hormone receptors, interferon and TNFα pathways, many of which have been linked to PML bodies. In addition, CBP has histone acetylation properties involved in chromatin remodelling. It is possible that the coordinating role of CBP occurs in PML bodies since, in addition to specific transcription factors, PML bodies were also reported to contain CBP (LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000). CBP is a dynamic component of PML bodies, unlike PML protein that is static (Boisvert *et al.*, 2001). There also appears to be a direct interaction between CBP and PML protein (Doucas *et al.*, 1999). However, the colocalisation of CBP with PML bodies is disputed, as this appears to be seen only with certain antibodies raised against CBP (LaMorte *et al.*, 1998). Furthermore, this observation is cell-type specific and may be dependent on the presence of oncogenic Ras (Pearson *et al.*, 2000). Here, a non-transcriptional role of CBP is also noted.

PML bodies are also reported to be involved in post-transcriptional control of gene expression. Colocalisation and coimmunoprecipitation of the eIF4E protein with PML bodies and protein has been noted (Cohen *et al.*, 2001). Direct interaction requires the RING domain of PML. Although eIF4E plays a role in translation initiation in the cytoplasm, it is thought to be involved in transport of mRNAs including cyclin D1 (Rosenwald *et al.*, 1995). Overexpression of PML inhibits eIF4E-dependent transport of cyclin D1 mRNA, resulting in nuclear accumulation of mRNA (Cohen *et al.*, 2001). More recently, a similar effect was seen with interferon treatment, while exposure to cadmium removes PML protein from the remaining eIF4E-containing bodies, with increased mRNA transport (Topisirovic *et al.*, 2002). Furthermore, the PML RING domain directly affects the eIF4E protein's ability to bind to the m7G cap of mRNA, and its subsequent transport function (Cohen *et al.*, 2001).

We can therefore see that PML bodies can either enhance or repress the effects of various transcription factors. Three possible mechanisms have been suggested for an indirect effect of PML bodies on transcription (Zhong *et al.*, 2000b). They are:

1. Titration. The PML body serves to concentrate transcription factors for neighbouring activity.
2. Modification. The PML body modifies and activates transcription factors. An example of this is p53, which undergoes acetylation and SUMOylation.
3. Compartmentalisation. The PML body sequesters transcription factors to repress their effects. An example of this is the sequestering of Daxx.

1.3.7.2 PML and cell cycle control

The PML protein has been shown to mediate growth suppression by affecting the cell cycle. PML -/- mouse embryonic fibroblasts (MEFs), were found to grow faster than PML +/+ or PML +/- cells, with more cells in S-phase and with increased ability to form colonies (Wang *et al.*, 1998a). Furthermore, PML -/- cells were unresponsive to the growth inhibitory effects of retinoic acid. Conversely, HeLa cells overexpressing PML showed inhibited cell growth and colony formation in agar, and an accumulation of cells in G1-phase and a delay into S-phase, in keeping with a decrease in the expression of cyclin E and cyclin-dependent kinase-2 (cdk2) (Mu *et al.*, 1997). Similar effects are seen in breast cancer cell lines if PML is overexpressed, where a block in G1 is associated with a decrease in cyclin D1 and cdk2 and an accumulation of hypophosphorylated Rb (Le *et al.*, 1998) (the role of pRb is discussed further in Section 1.3.7.4). Other cell lines, including CHO and NIH 3T3 show similar responses to PML overexpression (Koken *et al.*, 1995).

Studies have also shown that the RING, B-boxes and coiled-coil domains, as well as the NLS all contribute to the growth suppressor effect (Fagioli *et al.*, 1998). These results suggest that the growth suppression role of PML is related to the localisation of PML into PML bodies. Studies have also shown that PML directly interacts with checkpoint proteins, including chk2 (Yang *et al.*, 2002), pRb (Alcalay *et al.*, 1998) and p53 (Fogal *et al.*, 2000; Pearson *et al.*, 2000) (see Section 1.3.7.4).

1.3.7.3 PML and tumour suppression

In addition to the growth suppressor effect of PML protein/body, it also has a proposed tumour suppressor role that may not be mediated by cell cycle control. For example,

NIH 3T3 cells, which were transformed by the *neu* oncogene, revert to their original wild-type phenotype following overexpression of PML, including contact inhibition and anchorage-dependent growth (Liu *et al.*, 1995). This occurs without altering the proportion of cells in the different phases of the cell cycle. PML overexpression also inhibits transformation of rat embryo fibroblasts expressing H-ras and mutant p53 or H-ras and c-myc (Mu *et al.*, 1994). Transfection of PML into NB4 (APL) cells suppresses their ability to form colonies in soft agar. Conditioned medium from the transfected NB4 cells suppresses colony formation of untransfected NB4 cells suggesting soluble growth suppressor factors are produced. Transfection of PML in prostate cancer cells also suppresses their growth *in vitro* and decreases tumorigenicity in transplantation experiments in nude mice (He *et al.*, 1997).

The tumour suppressor effect of PML has been studied using animal models. PML -/- knockout mice do not have an increased rate of spontaneous tumour formation (Wang *et al.*, 1998a). Although these mice are fertile and viable, they are also more likely to succumb to infections preventing long-term follow-up. However, PML -/- mice develop more tumours when exposed to carcinogens or ionising radiation. When the skins of these mice are exposed to the tumour initiator dimethylbenzanthracine (DMBA) and the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), there is an increased incidence of skin papillomas and carcinomas, although the rate of progression from the papillomas to carcinomas is not increased. Furthermore, systemic DMBA treatment in these mice resulted in an increased incidence of tumours, mostly lymphomas and fibrohistiocytoma-like lesions.

1.3.7.4 PML, Ras, pRB and p53

The mechanism by which the PML protein and PML bodies play a role in growth and tumour suppression may be via complex interactions between Ras, p53 and Rb. This is illustrated in the increased accumulation of pRb and p53 in PML bodies when cell cycle arrest is induced by Ras in IMR90 fibroblasts, which also results in an increase in PML protein expression and PML body formation (Ferbeyre *et al.*, 2000). Furthermore, overexpression of PML alone produces the same result as Ras induction.

Subsequently, acetylation of the p53 protein was shown to occur in the presence of PML and CBP proteins in PML bodies, thus mediating Ras-induced senescence (Pearson *et al.*, 2000). Recruitment of p53 into PML bodies is mediated by the specific

PML3/PML IV isoform (Fogal *et al.*, 2000). However, PML IV alone is insufficient to induce senescence, as PML -/- cells with transfected PML IV remain resistant to its effects (Bischof *et al.*, 2002). p53 acts to transcribe target genes including proapoptotic *BAX* and cell cycle inhibitor *CDKN1A/p21*, and these effects are also mediated by the recruitment of p53 into PML bodies (Guo *et al.*, 2000). Interestingly, the PML gene itself is a transcriptional target of p53 (de Stanchina *et al.*, 2004).

Other factors involved in p53-mediated activities have also been shown to associate with PML, including HIPK2 (which also plays a role in Daxx activity) (D'Orazi *et al.*, 2002), and MDM2 (Wei *et al.*, 2003; Zhu *et al.*, 2003). As mentioned, p53 and MDM2 also accumulate in the nucleoli (Louria-Hayon *et al.*, 2003; Wei *et al.*, 2003). MDM2 and PML have been proposed to have opposing effects on p53, with p53 being activated by PML and repressed by MDM2 (Louria-Hayon *et al.*, 2003; Zhu *et al.*, 2003). However, MDM2 can also interact directly with PML protein in interferon-gamma treated cells (Wei *et al.*, 2003). The checkpoint protein, chk2 is thought to trigger apoptosis by both p53-dependent and independent pathways. Chk2 has been found in two studies to associate with PML bodies (Louria-Hayon *et al.*, 2003; Yang *et al.*, 2002). The protein is thought to act via phosphorylation of the PML protein, which leads to dissociation of the chk2 protein from PML bodies (Venere *et al.*, 2002; Yang *et al.*, 2002).

pRb controls the cell cycle by affecting E2F-mediated transcription of cell cycle genes including *CDC6*. The hypophosphorylated (inactive) form of pRb partially colocalises with PML bodies, and has been shown to interact directly with the PML protein (Alcalay *et al.*, 1998). As mentioned in the previous section (1.3.7.2), overexpression of PML results in a block in G1, which correlates with accumulation of hypophosphorylated Rb, as well as increased p53 expression in breast cancer cell lines (Le *et al.*, 1998). Since the growth suppressor effect of PML was seen even in Rb deficient cells, it could not be due only to Rb. However, a recent report suggested that PML overexpression-induced senescence was more likely to involve pRb, rather than p53 (Mallette *et al.*, 2004). In this study, PML-induced senescence was blocked by HPV E7 oncoprotein, but not by E7 mutants which were unable to bind pRb, or by dominant negative p53 mutants. pRb has been suggested to upregulate PML protein and PML body formation (Fang *et al.*, 2002).

1.3.7.5 PML and apoptosis

Apart from its interaction with p53, PML may activate apoptosis via other means. PML -/- mice and cells, for example, were found to be protected from different caspase-dependent apoptotic signals, such as Fas, TNF, ceramide, interferons and ionizing radiation (Wang *et al.*, 1998b). Likewise, APL cells showed similar resistance to these agents. In contrast, PML overexpression can induce apoptosis (Quignon *et al.*, 1998). Both studies suggested that regulation of apoptosis is unrelated to the transcriptional activity of PML, as there was no difference in gene expression between PML -/- and wild-type cells following irradiation. However, they differed in that while overexpression-induced apoptosis appeared to be caspase-3 independent (Quignon *et al.*, 1998), resistance to apoptosis following knockout of PML was related to insufficient caspase-1 and 3 activation. (Wang *et al.*, 1998b).

Further attempts to understand the underlying mechanism of the pro-apoptotic effect of PML determined that an intact RBCC domain is required (Borden *et al.*, 1997). In addition, Daxx (which has been shown to be a transcriptional repressor, Section 1.3.7.1), has been implicated in apoptosis. Daxx is thought to act by binding to the Fas receptor in the cell membrane and induction of the Jun N-terminal kinase (JNK) pathway. However, the localisation of Daxx into PML bodies, involving the C-terminal part of Daxx, is required for modulating Fas-induced apoptosis (Torii *et al.*, 1999; Zhong *et al.*, 2000c). It has been shown that both B and T lymphocytes of PML -/- mice become resistant to apoptotic factors, coinciding with an upregulation of Daxx (Wang *et al.*, 1998b; Zhong *et al.*, 2000c). Thus, PML bodies may to serve to activate the pro-apoptotic effects of Daxx. Consistent with this, Daxx, as well as Par-4 and ZIP kinase (which phosphorylates Daxx and Par-4), which are all required by IFNγ or arsenic-induced apoptosis, are all recruited into PML bodies following such treatments (Kawai *et al.*, 2003). One study also showed that the transcriptional repression activity of Daxx is diminished by its recruitment into PML bodies, as opposed to its apoptotic effects (Li *et al.*, 2000).

However, other studies have disputed this model of interaction between Daxx and PML bodies. For example, Daxx can act in the absence of PML, as shown by knockdown of PML by RNAi (Chen and Chen, 2003). Alternatively, it may be that PML bodies serve to sequester Daxx, as overexpression of HIPK2 resulted in release of Daxx from these bodies, as well as activation of the JNK pathway (Hofmann *et al.*, 2003). To further

complicate matters, Daxx itself can also have an anti-apoptotic effect, as Daxx knockdown showed apoptotic events in mouse cells (Michaelson *et al.*, 1999; Michaelson and Leder, 2003).

In addition to Daxx, other pro-apoptotic factors have been linked with PML bodies. For example, pro-apoptotic factor BAX, which gene is a target of p53 (see previous section), is also shown to colocalise with PML bodies, but the functional significance of this has not been elucidated (Quignon *et al.*, 1998). As mentioned, PML represses the signalling of TNFα via NF-kB, inhibiting this survival pathway and promoting apoptosis (Wu *et al.*, 2003b).

1.3.7.6 PML and telomere maintenance

A role for PML bodies in telomere maintenance was described in 1999 (Yeager *et al.*, 1999). In immortalised cell lines which lack telomerase activity, an alternative form of telomere maintenance was proposed, dubbed Alternative Lengthening of Telomere (ALT), which is achieved by homologous recombination (Henson *et al.*, 2002). In such ALT cells, larger PML bodies termed ALT-associated PML Bodies (APBs), were found to associate with the telomere-binding proteins hTRF1 and 2, replication factor A (RPA), RAD51 and RAD52 BRCA-1, Nijmegen Breakage Syndrome-1 (NBS-1), and Mre11 (Wu *et al.*, 2003a; Wu *et al.*, 2000). Furthermore, NBS-1 was found to be crucial for the formation of the larger bodies, which contained telomeric DNA repeat sequences (Yeager *et al.*, 1999). However, only 5% of ALT cells were found to have these aggregations of proteins in APBs. It was subsequently noted that APBs were found specifically in the G2/S-phases (Grobelny *et al.*, 2000; Wu *et al.*, 2000). In one of these studies, APBs were found to colocalise with BrdU-labelled replication foci (Wu *et al.*, 2000). Therefore, APBs may be formed at sites of telomere maintenance/synthesis during a specific period of S-phase.

1.3.7.7 PML and DNA repair

The role of PML bodies in DNA repair was first suggested from experiments showing that they contained the Bloom syndrome protein (BLM) (Zhong *et al.*, 1999b). BLM is a RecQ DNA helicase protein, which is involved in genomic stability. An absence of BLM is characterised by high levels of sister chromatid exchange (SCE) and an increased cancer predisposition. BLM becomes diffuse in APL cells, as well as PML -/- cells, which also have higher rates of SCE compared with PML expressing cells. This

suggests that PML protein is required for recruitment of BLM, as well as for proper functioning of BLM. The expression and localisation of BLM varies widely, with high expression in lymphoid cell types and proliferating tissues (Turley *et al.*, 2001). The BLM protein also shows a cell cycle dependent distribution. In S-phase, in addition to its colocalisation with PML bodies, BLM is also found in the nucleolus (Yankiwski *et al.*, 2000). It has been proposed that the association with the nucleolus, which occurs via the C-terminus of the protein (as compared with N-terminus involved in PML body localisation) is more important for genomic stability (Yankiwski *et al.*, 2001). Furthermore, in late S-phase, BLM-containing PML bodies interact with late replicating foci, telomeres and RPA (Yankiwski *et al.*, 2000). This association is increased in ALT cells suggesting that BLM is involved in telomere maintenance as well (see previous section). BLM also interacts with p53. The accumulation of BLM in PML bodies is facilitated by p53, and BLM cooperates with p53 in p53 –mediated apoptosis, but not in p53-mediated transcription (Wang *et al.*, 2001).

The redistribution of PML bodies to sites of DNA damage has also been reported in various studies, and many other DNA repair proteins have been shown to associate with these PML bodies following DNA damage. In addition to BLM, they include p53, MDM2, Mre11, RPA, RAD51, and WRN (Bischof *et al.*, 2001; Blander *et al.*, 2002; Carbone *et al.*, 2002; Kurki *et al.*, 2003). Many of the proteins are also recruited into APBs (see previous section), as DNA repair and ALT may both involve repair machinery such as NHEJ and homologous recombination.

1.3.7.8 PML and DNA replication

A direct role of PML bodies in DNA replication, if any, has not been studied in detail, and has even been dismissed (Borden, 2002). The role of PML bodies in DNA replication will be discussed further in Chapter 5.

1.3.7.9 PML and immune function

The upregulation of PML protein and PML bodies by interferons and in inflamed tissues has been described above. Other components of PML bodies, such as Sp100 and ISG20, (Gongora *et al.*, 1997; Guldner *et al.*, 1992), are likewise upregulated by both type I and II interferons. These findings suggest that PML bodies are involved in the cellular defence or the immune system within the cell.

Such a role was shown in experiments where overexpression of PML protein in a variety of cells increased resistance to viral infections, including Ebola virus, vesicular stomatitis and influenza A, though not encephalomyocarditis virus (Bjorndal *et al.*, 2003; Chelbi-Alix *et al.*, 1998). This anti-viral effect was apparently mediated by the coiled-coil domain of the PML protein. Conversely, PML -/- mice were more susceptible to developing botryomycotic lesions (Wang *et al.*, 1998a). However, PML -/- cells (with or without IFNα treatment) did not appear to be less (or more) resistant than controls to viral infections, including the vesicular stomatitis virus mentioned above, as well as herpes simplex virus and adenovirus (Lavau *et al.*, 1995). In addition, the hypothesis for a defence response by PML is disputed by the fact that many different viral infections require the presence of PML bodies for replication of virions (see Section 1.3.8.3).

How do PML bodies confer cellular immunity? In cell lines, a direct anti-viral effect cannot be ruled out, although the PML protein may also modify other cellular proteins, resulting in viral resistance (Chelbi-Alix *et al.*, 1998). In whole organisms, one possible mechanism is via induction of the MHC antigen presentation pathway (see Chapters 3 and 4). PML bodies also recruit immunoproteasomes, which are involved in protein degradation and antigen presentation (see Section 1.3.8), but it is not clear if this confers a protective function.

1.3.7.10 PML bodies as nuclear depots for protein degradation

The recruitment of the various proteins to PML bodies has given support for roles in diverse nuclear processes (Ruggero *et al.*, 2000; Seeler and Dejean, 1999). A model has therefore been proposed for PML bodies to serve as a nuclear depot, coordinating the accumulation and release of regulatory proteins from all these pathways in response to external stimuli (Negorev and Maul, 2001). There is much appealing evidence to support this interpretation. As mentioned in Section 1.3.5, many proteins accumulate in PML bodies only when overexpressed or in the inactive form. Foreign proteins, such as the lac repressor or GFP-fused proteins, when introduced into cells also become associated with PML bodies (Baumann *et al.*, 2001; Tsukamoto *et al.*, 2000). Mutant polyglutamine proteins, such as Huntingtin protein (in Huntington's chorea) and ataxins (in spinocerebellar ataxia) likewise associate with PML protein, and form larger inclusion bodies (Skinner *et al.*, 1997; Yasuda *et al.*, 1999). (See Section 1.3.8.4 for more details regarding these mutant proteins.)

PML bodies have recently been shown to associate with proteasome subunits, which are multiprotein structures involved in the cellular degradation of proteins (Baumann *et al.*, 2001; Fabunmi *et al.*, 2001). (See Section 3.1.6 for a review of the structure of proteasomes.) Briefly, the core 20S subunit of the proteasome can combine with the PA28 subunit to form the immunoproteasome, involved in antigen presentation on the cell surface. Although the 20S subunit appears to be located predominantly in the cytoplasm (Mattsson *et al.*, 2001), the PA28 subunit is found in a proportion of PML bodies, as well as in the cytoplasm (Fabunmi *et al.*, 2001). Treatment of cells by IFNγ results in the relocation of the 20S subunit into PML bodies, as well as an increase in the proportion of PML bodies containing PA28 (Fabunmi *et al.*, 2001). This suggests that that PML bodies may be sites of immunoproteasome formation. However, degradation of exogenous proteins may also occur in immunoproteasome-containing PML bodies, as these bodies recruit foreign proteins. Furthermore, the presence of proteasomes in PML bodies is enhanced by arsenic, which also results in increased breakdown of cellular proteins (Lallemand-Breitenbach *et al.*, 2001). These studies suggest that PML bodies may be sites of protein degradation, and possibly also antigen presentation, for such foreign or mutant proteins.

The evidence so far suggests a passive sequestration or a degradation role for these PML bodies. Instead, there is evidence that the recruitment of various proteins into PML bodies can actually enhance their activities. Numerous other examples have already been given in the Sections above (e.g. BLM, p53, β-catenin).

What are the mechanisms that allow proteins to be recruited or released from PML bodies, and are these important for the proposed regulatory role of PML bodies? These questions have been partially answered in the previous sections. As discussed, the PML protein itself has been shown to be essential for the recruitment of a number of proteins, involving the RING structure and SUMOylation of PML (Zhong *et al.*, 2000a). In contrast, the release of proteins associated with PML bodies can also be demonstrated experimentally, but only following external stresses, such as heat shock (Nefkens *et al.*, 2003). Physiological conditions for recruitment and release of factors from PML bodies is an area that requires more studies.

1.3.8 PML bodies and disease states

The study of PML bodies is complicated by their diverse proposed role(s). This is further compounded by the pathological conditions that PML bodies are associated with, which may nevertheless be important in understanding their biology.

1.3.8.1 Acute promyelocytic leukaemia

Apart from being clinically important, APL is also useful as a model for studying the possible functions of the PML protein. In contrast to the PML protein, the parent RARα protein has defined functions. It has a structure similar to other nuclear steroid receptors, containing six evolutionarily conserved domains (reviewed in Melnick and Licht, 1999). Upon stimulation by all-trans retinoic acid (ATRA), RARα heterodimerises to RXR, and binds to retinoic acid response elements (RARE) in promoter sites of target genes to activate them. In addition, RARα-RXR interacts with several cofactors. In the absence of ATRA, RARα binds to corepressors N-CoR and SMRT, and HDAC complexes. With ATRA, binding of co-activators, including CBP occurs (Kamei *et al.*, 1996; vom Baur *et al.*, 1996). One such cofactor is PML protein (Wang *et al.*, 1998a; Zhong *et al.*, 1999a). RARα-mediated transcription is thought to be responsible for myeloid differentiation. A subcloned cell line of the HL60 leukaemic cell has a mutation of RARα which results in a myeloid differentiation block, which is reversible by transfecting wild-type RARα (Collins *et al.*, 1990). The multiple targets of RARα include genes encoding cell cycle regulators, transcription factors, cytokines and cytokine receptors and cell surface adhesion molecules (Melnick and Licht, 1999).

In APL, a t(15;17) translocation results in the formation of the PML-RARα and (the lesser studied) RARα-PML fusion products (Goddard *et al.*, 1991). There are three recognised breakpoints in the *PML* gene, all occurring after the RBCC motif-encoding exons (Pandolfi *et al.*, 1992). The PML-RARα protein acts in a dominant manner, disrupting the functions of the RARα and PML proteins encoded by the remaining allele. The PML-RARα protein retains both the DNA- and ligand-binding domains of RARα, but differs in its kinetics of binding to cofactors and the target elements. It can therefore affect the retinoic acid-mediated transcription pathway by several different mechanisms (Perez *et al.*, 1993).

PML-RARα also disrupts the normal distribution of PML bodies (Daniel *et al.*, 1993), resulting in a microspeckled pattern of more than a hundred small (0.1 μm) dots. This is due to the heterodimerisation between PML-RARα and wild-type PML from the remaining allele, via their coiled coil domains. Other PML body-associated proteins are also delocalised, and some proteins such as Sp100, but not others (e.g. Daxx) colocalise in the microspeckles (Koken *et al.*, 1994; Zhong *et al.*, 2000c). Because loss of PML bodies results in disorders in cell growth and tumorigenicity, it has been suggested that these spatial effects also contribute to leukaemogenesis (Ruggero *et al.*, 2000). For example, APL cells (similar to PML -/- cells) show resistance to Fas, TNF and IFN-induced apoptosis (Wang *et al.*, 1998b).

Treatment of APL with high doses of ATRA induces myeloid differentiation and a clinical remission of leukaemia. ATRA acts in a number of ways. Firstly, higher than physiological doses of ATRA causes the dissociation of PML-RARα from the corepressor complex, resulting in transcriptional activation similar to RARα (Guidez *et al.*, 1998). Secondly, ATRA treatment also degrades PML-RARα, by a mechanism involving the proteasome (Yoshida *et al.*, 1996). Cleavage of PML-RARα occurs in the RBCC motif, leading to an inability of the two fragments to bind the wild-type copy of PML. A normal PML body distribution is seen in ATRA-treated APL cells (Daniel *et al.*, 1993). A similar effect is also seen when APL cells are treated with arsenic compounds (Zhu *et al.*, 1997). Here, PML-RARα is rapidly incorporated to the newly formed PML bodies, where it is degraded.

APL can also arise from other chromosome translocations involving *RARA* but not always *PML*. Other fusion partners of RARα include the *PLZF* zinc finger gene [t(11;17)(q23;q21)], *NuMA* (nuclear mitotic apparatus) gene [t(11;17)(q13;q21)] and *NPM* (nucleophosmin) gene [t(5;17)(q32;q21)] (Grimwade *et al.*, 1997; Redner *et al.*, 1996; Wells *et al.*, 1997). These fusion products do not alter the normal PML body pattern, indicating that PML body disruption is not required for APL pathogenesis.

1.3.8.2 PML and other cancers

PML protein/bodies (without RARα) have been implicated in other tumours besides APL. Section 1.3.7 has dealt with the role of PML in growth and tumour suppression. Here, the expression of PML in different tumours will be discussed.

Different neoplasms have been shown to vary widely in PML expression as detected by immunohistochemistry, with increased expression in Hodgkin's disease, renal cell carcinoma and oesophageal carcinoma, but decreased expression in gastric, colon and breast carcinomas (Gambacorta *et al.*, 1996; Koken *et al.*, 1995; Terris *et al.*, 1995). More recently, microarray analysis together with immunohistochemistry (IHC) and Northern blotting for PML expression showed that most carcinomas have decreased expression of PML, with the exception of adrenal and thyroid carcinomas (Gurrieri *et al.*, 2004).

PML is also found to be expressed in only specific histological subtypes of tumours. For example, papillary thyroid carcinomas have overexpression of PML, while follicular carcinomas show negative or weak antigenicity (Gambacorta *et al.*, 1996). PML expression is also increased in squamous or adenocarcinoma of the lung, but decreased in small cell lung cancer (Zhang *et al.*, 2000). Uniquely, hepatocellular carcinoma (HCC) cells show a granular cytoplasmic pattern of PML. However, this may be a consequence of the altered expression in (premalignant) cirrhosis of the liver (Chan *et al.*, 1998; Yoon and Yu, 2001). The upregulation of PML bodies in HCC was not found to be mirrored in liver metastases from colonic primaries. Furthermore, tumour sections of HCC also showed increased PML expression in the cells surrounding the tumour, including lymphocytes, macrophages and fibroblasts. Some studies also suggested that the level of PML expression correlates with the degree of dysplasia in atypical breast hyperplasia and cervical intraepithelial neoplasm. However, in one study, when breast tumours invaded beyond the epithelial basal lamina, PML expression decreased again (Koken *et al.*, 1995). Loss of PML expression is also related to higher grades of tumours, invasiveness and presence of metastasis (Gurrieri *et al.*, 2004). Decreased PML expression also seems to correlate with downregulation of genes involved in MHC antigen presentation (Cabrera *et al.*, 2004). Conversely, increased expression of PML protein in neuroblastoma cell lines correlated with a more differentiated phenotype (Yu *et al.*, 2003) and response to retinoic acid treatment (Yu *et al.*, 2004).

Cell growth and tumour suppression arising from overexpression of PML has prompted investigations into the possibility of PML in gene therapy. Virus mediated transfection of PML protein into prostate, breast and bladder cancer cell lines has led to growth suppression and decreased tumorigenicity (He *et al.*, 1997; He *et al.*, 2003; Le *et al.*, 1998). Although the role of PML in MHC class I expression is controversial (see above,

Section 1.3.7 and Chapter 4), upregulation of the MHC class I genes has been seen in lung cancer cell lines following transfection of PML (Chang *et al.*, 2002).

These gene therapy studies are consistent with the observation that PML expression correlates with a malignant phenotype. It remains to be seen whether differential expression of PML bodies is unique to the transformation of different neoplasms, or whether it reflects underlying differences in normal expression of PML in different tissues. Ultimately, the yet unanswered question is whether altered PML expression is an important step in tumorigenesis, or simply a bystander or secondary effect.

1.3.8.3 PML and viral infections

An increasing number of viral infections appear to involve PML bodies. A summary of viral-PML interactions is shown in Table 1.5. Several of these viruses show similar patterns of association with PML bodies. For example, early viral proteins of CMV and HSV-1 disrupt the pattern of PML bodies. Subsequently, the replication and transcriptional activities of these viral genomes are found in the vicinity of the altered PML bodies. It has been proposed that viruses target PML bodies to (a) hijack PML proteins which can be used for viral replication and transcription and/or (b) disrupt the defence mechanisms of PML bodies (Moller and Schmitz, 2003).

Therefore, PML bodies appear to participate in viral infection, despite the possible anti-viral and immune roles of PML bodies (see Section 1.3.7). Could it be, therefore, that a battle for control exists in PML bodies between defence mechanisms and viruses? Is the resistance conferred by PML limited only to specific infections? Alternatively, could the immune function of PML bodies and/or protein be limited only to mice, or even whole organisms, as opposed to cell lines? It also remains to be seen if the role of PML bodies in infection can be exploited clinically.

1.3.8.4 PML and neurodegenerative diseases

As discussed in Section 1.3.7.10, PML bodies are implicated in the pathogenesis of a rare neurodegenerative disorder, spinocerebellar ataxia type 1 (SCA1) (Skinner *et al.*, 1997). This disease is caused by mutations in the *SCA1* gene, resulting in a mutant gene product, ataxin-1, which contains expanded poly-glutamine tracts. Mutant ataxin-1 aggregates with PML protein to form enlarged (but fewer) PML bodies in cerebellar Purkinje cells. This is also seen when mutant ataxin-1 was transfected into COS-1 cells.

Table 1.5. Viral proteins associating with PML bodies.

Virus	Component associated with PML bodies	Function	References
HSV-1	ICP0 (Vmw110)	Disrupts PML via SUMO	(Everett and Maul, 1994)
	ICP4	Transcription regulator	(Everett *et al.*, 2003)
	HSV-1 DNA	Replication sites	(Ishov and Maul, 1996)
CMV	IE1, IE2	Disrupts PML via SUMO, viral replication	(Ahn and Hayward, 1997)
	Pp71	Viral transcription via Daxx	(Marshall *et al.*, 2002)
EBV	EBNA-5	Involved in latency and transformation, associates with p53.	(Szekely *et al.*, 1996)
	BZLF-1	Disrupts PML	(Adamson and Kenney, 2001)
HHV-6/8	Early protein K8	Does not disrupt PML	(Katano *et al.*, 2001)
Ad5	E4ORF3, E1A	Redistributes PML into sites of viral replication	(Carvalho *et al.*, 1995)
SV-40	T-antigen	Adjacent to PML bodies associates with p53	(Ishov and Maul, 1996)
LCV	Z	Relocates PML protein to cytoplasm, targets P protein in PML bodies for replication	(Borden *et al.*, 1998)
Lassa	Z	As above	(Kentsis *et al.*, 2001)
HTLV-1	Tax	Oncoprotein	(Desbois *et al.*, 1996)
HPV	L2	Capsid protein	(Day *et al.*, 1998)
Hep B	polymerase	Replication	(Choi *et al.*, 2003)
Rabies	P proteins	Unknown	(Blondel *et al.*, 2002)

HSV-1 –Herpes Sim plex Virus-1, CMV –Cytom egalovirus, EBV –Ebstein-Barr Virus, HHV –Hu man Herpesvirus, Ad5 –Adenovirus 5, LCV - Lym phocytic choriomeningitis virus, SV-40 – Sim ian virus 40, HTLV-1 – Hum an T-cell Lymphotropic Virus-1, HPV – Human Papillomavirus, Hep B –Hepatitis B virus.

In contrast, wild-type ataxin-1 is normally present in a speckled pattern, which does not colocalise with PML bodies. Mouse models in which mutant forms of *SCA1* have been introduced have shown that loss of normal nuclear localisation, rather than aggregation of Ataxin-1, is required for the disease to develop (Klement *et al.*, 1998). It remains to be seen if the association of ataxin-1 and such other mutant proteins are a consequence of the disease, or if direct roles exist in the clinical presentation.

Since this initial discovery, several other related disorders, including Huntington's chorea and other SCA types, with polyglutamine abnormalities were also found to have similar effects on PML bodies (Takahashi *et al.*, 2003; Yasuda *et al.*, 1999).

From this review of PML protein/bodies, we see that they are implicated in multiple pathways, but no definite functions have been proven. Furthermore, the roles that have been suggested for PML bodies are diverse and sometimes bear no connection to each other. Is the PML body an elephant that we have so far been exploring blindly?

1.4 Aims of the thesis

In the nucleus, the functions of various sub-structures can often be revealed by understanding their spatial positions and interactions with other structures. With regard to PML bodies, earlier experiments have suggested that they associate preferentially with certain genomic loci (Shiels *et al.*, 2001; Sun *et al.*, 2003).

My major aim was to elucidate the basis of the association of PML bodies with the genome. The main approach was to use microscopic imaging to characterise the spatial arrangement of PML bodies. Although this is a descriptive analysis, these structures would be studied in a physiological environment. A statistical model which had been developed and applied previously, but which required further validation, was used. This would enable me to identify the main characteristics that determine an association between PML bodies and specific genomic regions. I also wished to determine whether PML-genome associations have any functional consequences, in terms of activity of the genomic regions. In particular, the spatial associations were compared with transcription of various genes, principally using reverse transcriptase-polymerase chain reaction (RT-PCR). Finally, the spatial association of PML bodies with sites of DNA replication were investigated to determine if PML bodies may play a role in replication. It was hoped that an integrated model of PML body organisation could be proposed.

Chapter 2: Materials and Methods

2.1 General cell culture, treatments and transfections

2.1.1 Culture of established cell lines

Cell lines were grown on ungelatinised tissue culture plasticware. All cells were grown at 37 °C, with either 5% or 10% CO_2, and 100% humidity.

MRC5 male and WI-38 female human primary fibroblasts (both having a normal diploid karyotype) were cultured as monolayers in RPMI-1640 media supplemented with 10% Foetal Calf Serum (FCS) and 2 mM glutamine, and with streptomycin and penicillin V as antibiotics. HeLa ovarian carcinoma cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with FCS, glutamine and antibiotics as above. CAKI-1 (HTB-46) and CAKI-2 (HTB-47) renal cell carcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown as monolayers in McCoy's 5a medium with supplements as above. NB4 (APL) cells and AHB (EBV-transformed human lymphoblastoid B-cell line with two normal copies of chromosome 6) were grown in suspensions of RPMI-1640 with supplements as above.

Cells grown as monolayers were passaged when they reached confluency, usually every third day. Cells were washed with phosphate buffered saline-A (PBS-A) (see Appendix A for recipes of common solutions), and then were detached with 1 ml trypsin/versene. They then incubated at 37 °C until fully digested (typically ~5 minutes), and the trypsin digestion terminated by the addition of an excess of the appropriate growth medium with FCS. Cells were divided between two new flasks at 30 - 50% confluency. For microscopy, cells were seeded on chamber slides (Labtek) 24 to 48 hours prior to fixation. Any treatment of cells was performed on these chamber slides.

Cells grown in suspension were sub-cultured when they reached approximately 10^6 cells/ml, usually every third day. Cells were centrifuged at 300 x *g* for 5 minutes. The pellet was resuspended in 2 ml of fresh medium and divided between two new flasks each containing 29 ml of fresh medium. This gave approximately 5 x 10^5 cells/ml. For microscopy, suspension cells were incubated for 1 hour in medium on polylysine coated slides prior to fixation.

2.1.2 Freezing and thawing of cells

Aliquots of ~10^6 cells were frozen in a volume of 1 ml. Briefly, cultures were trypsinised and resuspended at ~2x10^6 cells/ml in growth medium. An equal volume of 2x freezing medium (10% dimethyl sulphoxide, DMSO in growth medium or foetal calf serum) was added dropwise to the cells, with continual mixing. The aliquoted cells were placed at –70 °C in a Cryo 1 °C Freezing Container (Nalgene) and subsequently stored under liquid nitrogen. Cells were thawed rapidly at 37 °C in a waterbath, and immediately placed in the appropriate growth medium in a 25 cm^2 flask. Once the cells had adhered to the growth surface they were washed with PBS-A to remove all traces of DMSO and then fed.

2.1.3 Treatment of cells

1. Heat shock of MRC5 cells was performed at 44 °C for 1h on a heating block (Jolly *et al.*, 1997).
2. Expression of MHC and other genes in different cell lines was induced using the cytokine IFNγ (Boehm *et al.*, 1997). Briefly, cells were incubated for 2 to 24 h with 200 U/ml (final concentration) of recombinant human IFNγ (R&D Systems).
3. Differentiation of NB4 cells was induced with 1 μM ATRA (final concentration) for 48h (Lee *et al.*, 2002).
4. Transcription foci were labelled by adding fluorouridine (FU, Sigma) to the culture medium at a final concentration of 10 μM, 10 to 20 minutes immediately prior to fixation (Boisvert *et al.*, 2000).
5. Replication foci in cells in S-phase were labelled by adding BrdU (Sigma) to the culture medium at a final concentration of 100 μM for 1hour, and fixed immediately. To label nascent DNA at a time prior to fixation, cells which were pulsed with BrdU were then washed with new medium 3 times and incubated for 1 to 7 hours with medium without BrdU before fixation (see Figure 2.1.A) (Tashiro *et al.*, 2000).
6. To visualise replication foci at two different times, cells were first pulsed with BrdU for 1 hour, washed, and after an interval of 1 to 4 hours, labelled with either iododeoxyuridine (IdU), or fluorescein-/Texas Red-deoxyuridine triphosphate (FITC-/TR-dUTP, PerkinElmer) using the scratch labelling method (Schermelleh *et al.*, 2001; Zink *et al.*, 1998). Essentially, labelled dUTP was added to cells in chamber slides at a concentration of 25 μmol/ml in medium (with volumes sufficient to just cover the cells). A 25-gauge needle was scored across the slide

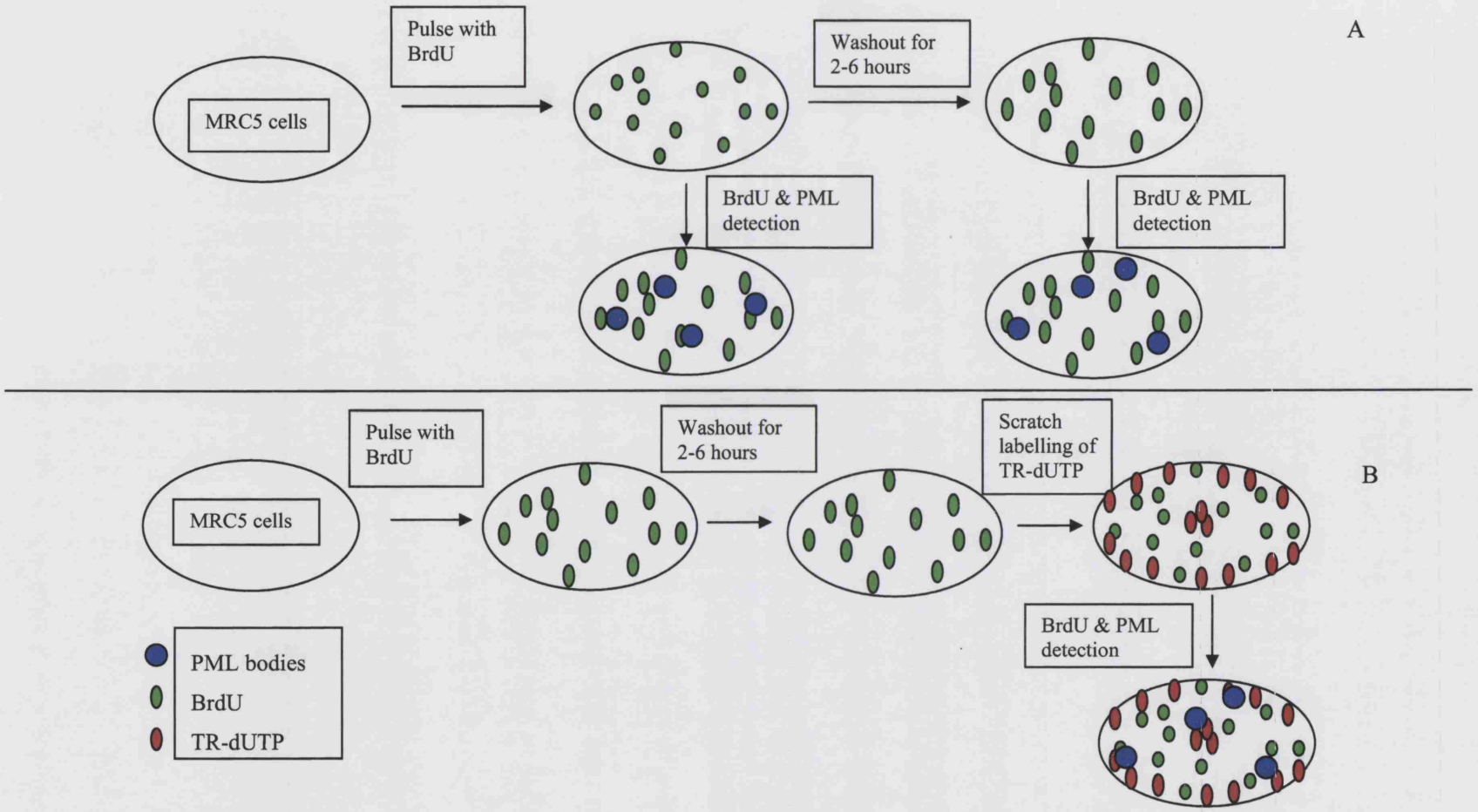

Figure 2.1. Diagram illustrating experiments using washout and dual labelling of replication foci.
A: Sequence of events involved in washout experiments using MRC5 cells.
B: Sequence of events involved in dual labelling of replication foci using MRC5 cells.

surface, at approximately 1 mm intervals. Cells were incubated for 2-3 minutes, and the dUTP was diluted 3 fold in further medium. Cells with IdU or fluorochrome-dUTP were then incubated for a further hour. Cells may also be labelled with IdU or fluorochrome-dUTP first, followed by BrdU (see Figure 2.1.B).

7. To synchronise cells at the G1/S boundary, a double block by thymidine was applied (Detke *et al.*, 1979). Subconfluent cultures were first treated with 2 mM thymidine for 18 hours to arrest cells at G1/S-phase (with some arrested in mid-S-phase). The thymidine was then washed out, and cells grown in normal medium for 8 hours to allow them to progress out of S-phase. Cells were then treated a second block of 2 mM thymidine for 18 hours.

2.1.4 Overexpression of PML protein by transient transfection

Using total RNA extracts from MRC5 cells (see later), reverse transcribed to complementary DNA (cDNA), PML transcripts were amplified using primers designed against the PML isoform IV (previously PML-3), see Appendix B.1. The forward primer was designed to include the start codon, and to be in frame with the insertion region of the vector (see later). The reverse primer was designed beyond the stop codon. The PCR reaction was performed as described in Section 2.4.3. The PCR product was separated on 1.5% agarose gels (1.5% w/v agarose in TAE (tris(hydroxymethyl) aminomethane, Tris-acetate-ethylenediaminetetraacetic acid, EDTA) buffer with 1 μg/ml ethidium bromide), together with a 1 kb DNA ladder (Amersham). Gels were run at 120 V in 1xTAE buffer and visualised on an ultraviolet (UV) transilluminator.

Following electrophoresis, gel slices corresponding to DNA of approximately 2,500 bp identified using a 1 kb DNA ladder (Amersham), were excised under UV light. DNA was extracted from the gel slices using Qiaquick columns (Qiagen), following the manufacturer's gel extraction protocol. Purified DNA was eluted from the silica-based solid support using 30 μl of deionised water.

The expression vector used was from the pcDNA3.1/V5-His TOPO vector kit (Invitrogen). This contains a CMV promoter that allows constitutive expression of the vector insert. This vector has been linearised with a thymidine 3' overhang on both ends, attached to topoisomerase enzymes. This vector allows insertion of a PCR product (made with Taq polymerase and containing a 5' adenosine overhang) without the need for restriction enzyme digestion and re-ligation. Two μl of the purified PCR product

was admixed with 4 µl of the vector and 1 µl of salt solution (1.2 M NaCl, 0.06 M MgCl2), and incubated for 30 min at room temperature. Two µl of the mixture was then added to TOP10 chemically competent E coli. Transformation of E coli was performed chemically, by heat shock at 44 °C for 30 sec. The bacteria were then incubated in SOC medium at 37 °C for 1 hour, and plated onto LB agar plates containing 100 µg/ml of ampicillin. As a positive control, a vector containing the LacZa gene supplied in the kit was used.

Following incubation of the agar plates at 37 °C overnight, single colonies were picked and grown in 100 ml of L-Broth overnight. Plasmid DNA was then isolated by DNA extraction (midipreps, Qiagen; refer to next section). Different colonies were selected and subjected to sequencing to confirm the presence and orientation of the inserts. PCR cycle sequencing using fluorescent dye-labelled terminators during enzymatic extension reactions was employed. 2 µl DNA template was added to 8 µl of ABI prism terminator ready reaction mix (PerkinElmer), and 3.2 pmol of reverse BGH primer (Invitrogen), and made up to 20 µl with deionised water. Following PCR, unincorporated nucleotides were removed by ethanol precipitation, and cycle sequencing reactions were loaded on an automated sequencing machine.

The samples were further quantified by UV spectrometry, with an absorbance of one A260 optical density (OD) unit corresponding to 50 µg/ml. The vector was transfected into MRC5 cells transiently (using Superfect reagent, Qiagen). Five µg of the vector was mixed with RPMI medium without antibiotics or FCS (to a final volume of 150 µl). Thirty µl of the Superfect liposomal reagent was then added, mixed and incubated for 15 min. One ml of RPMI medium containing FCS, penicillin and streptomycin was added next and the whole volume placed on pre-washed MRC5 cells in a chamber slide (bottom surface area 8 cm^2) for microscopy. Alternatively, volumes were scaled up three-fold for transfection in MRC5 cells in a small 25 cm^2 tissue culture flask. Cells were incubated for 3 hours before they were washed. Cells were grown for a further 48 to 72 hours prior to immunofluorescence, RNA or protein analysis.

2.1.5 RNA interference (RNAi) treatment

RNAi treatment on MRC5 cells was performed using 21-nucleotide small interfering RNA (siRNA) strands as previously described (Bruno *et al.*, 2003). Several siRNA

strands were designed or based on those used by Bruno *et al* (Appendix B.1). RNAi oligonucleotides were custom ordered either from Qiagen or Invitrogen. In addition, a pre-designed non-silencing control siRNA oligonucleotide was obtained from Qiagen.

Transfection of siRNAs was tested using the Transmessenger reagent (Qiagen) or the Oligofectamine reagent (Invitrogen). The oligofectamine reagent was found to be more efficient and will be described here. Ten µl of 20 µM stock siRNA was mixed with 160 µl of RPMI without antibiotics. At the same time, 3 µl of Oligofectamine was added to 12 µl of RPMI without antibiotics in a separate tube, and incubated at room temperature for 10 min. The two solutions were then admixed and incubated for 20 min. This mixture was then added to a chamber slide containing washed MRC5 cells and 800 µl of RPMI without antibiotics or FCS. The cells were then incubated at 37 °C. After 4 hours, 500 µl of RPMI containing antibiotics and 30% FCS was added. Cells were then grown for 24-96 hours prior to fixation for immunofluorescence. For RNA or protein analysis, cells were grown in small 25 cm^2 tissue culture flasks, and the volumes of the solutions scaled up by a factor of three.

2.2 Immunofluorescence, ImmunoFISH and Microscopy

2.2.1 DNA probes

The **LMP/TAP** locus in the MHC was detected using a pool of cosmids HA14, U15, U10, M4 containing the *LMP2/PSMB8, LMP7/PSMB9, TAP1* and *TAP2* genes. The ***C4B*** gene was detected using cosmids CH122, BF23 and K101. The ***HLA-A*** gene was detected using cosmids C0426 and A0622. All MHC probes were gifts from S Beck and J Trowsdale. A subsection of the **6p24** region (near the *TFAP1* gene) was detected using pooled cosmids A9.5, B5.10, B10.10, B12.10, E11.2, F1.6 (gift from J Ragoussis). The Histone **H2-4** region in band 6p22 was detected using pooled cosmids A1037, H2317, N1948 (gift from J Trowsdale). The **PBX1/NOTCH4** locus in the MHC class III region was detected using bacterial artificial chromosomes (BACs) probes RP11-449A12 and RP11-568L21, derived from Dr Pieter de Jong (Roswell Park) and provided by the Sanger Institute. Probes for loci on other chromosomes were BACs and P1 artificial chromosomes (PACs), also derived from Dr Pieter de Jong, see Appendix B.2. All probes were tested by PCR with primers designed for the genes encoded, as well as by FISH mapping on metaphase spreads.

The whole chromosome territories of chromosomes 1, 6, 9, 18 and 19 and X were detected using commercial biotinylated satellite probes and red- or green-fluorescent whole chromosome paints (Appligene Oncor). Xp and Xq arm probes (biotin and digoxigenin labelled respectively) were obtained from Cambio Ltd. The centromeres of chromosomes 1, 6, 9, 13 and 21, 14 and 22, 15, 18, 19 and X were detected using commercial biotinylated satellite probes (Appligene Oncor). Since the satellite probe for chromosome 19 cross-hybridises to chromosomes 1 and 6, it was hybridised together with the chromosome 19 paint to allow identification of the chromosome 19 centromere.

2.2.2 Antibodies for immunofluorescence

Rabbit polyclonal anti-PML (gift from P Freemont), goat polyclonal anti-PML (N19, Santa Cruz) were used to detect PML nuclear bodies. To detect IdU or BrdU, and FU incorporated into newly replicating DNA and new RNA transcripts respectively, different anti-BrdU (Abcam (rat), Boehringer Mannheim Corp and Sigma (both mouse)) were used. The anti-BrdU antibody from Boehringer is known to detect both IdU and BrdU, while the antibody from Abcam detects BrdU specifically. The anti-BrdU antibody obtained from Sigma is known to detect fluorouridine. Monoclonal mouse anti-splicing factor SC-35 (Sigma) was used to detect SC-35 domains.

Secondary antibodies used were anti-mouse alexa 488 and alexa 546 (either raised in goat or chicken), chicken anti-rat alexa 546, chicken anti-rabbit alexa 488 and 546, and donkey anti-goat alexa 546 and alexa 633 (all Molecular Probes), and goat anti-rabbit cy5 (Amersham Pharmacia). All antibodies were used at a dilution of 1:100.

2.2.3 Bacterial growth and DNA extraction

Bacterial glycerol stocks or liquid cultures were streaked on LB-agar plates and grown overnight at 37 °C to obtain single bacterial colonies. For liquid cultures, 5 ml LB-broth was inoculated with a single bacterial colony and shaken at 200 revolutions per minute (rpm) overnight at 37 °C. Where appropriate, media were supplemented with antibiotics. The starter culture was then diluted (1/500 to 1/1,000) into 100 ml selective L-broth and incubated overnight at 37 °C with vigorous shaking. Bacteria were harvested by centrifugation at 6,000 x *g* for 15 minutes using a Beckman JA-10 rotor. Bacteria were pelleted from suspension culture and plasmid DNA prepared essentially

by the alkaline lysis method using Qiagen Midi or Maxi preps, according to the manufacturer's instructions.

The resulting DNA pellet was allowed to air dry and then resuspended in 50-80 µl of TE (Tris-EDTA) buffer pH 8.0. DNA was quantified by UV spectrometry, with an absorbance of one A260 OD unit corresponding to 50 µg/ml.

2.2.4 Labelling of probes

One to 1.5 µg of each probe was labelled by nick translation with biotin-14-dATP (BRL Bionick kit) or digoxigenin-11-dUTP (Boehringer) according to the suppliers' instructions. Briefly, the following were added to ~500 ng clean probe DNA (plasmid, cosmic, phage or BAC): 2 µl 10x nick translation salts; 2.5 µl each 0.5mM dATP, dCTP, dGTP; 2.5 µl biotin-16-dUTP/ 1 µl digoxigenin-11-dUTP + 1.5 µl dTTP; 1 µl 1:500 fresh dilution of deoxyribonuclease (DNase) I (1 mg/ml, Boehringer); made up to a volume of 19 µl with distilled water. The mixture was mixed, 1 µl DNA polymerase I (10 U/µl Gibco-BRL) added, and the reaction incubated at 15 °C for 90 minutes. The reaction was stopped by the addition of 2 µl 0.2M EDTA/ 1 µl 5% sodium dodecyl sulphate (SDS). Labelled probes were stored at –20 °C. To ensure that the probe was in the preferred size range (150 - 500 bp), 5 µl of the labelled mixture was run on a 0.8% agarose gel.

Between 200-300 ng of probe was combined with 5 µg of Cot1 DNA and 1 µg of salmon sperm DNA (Gibco BRL) and precipitated using 2 volumes of 100% ethanol and incubation at –20 °C overnight. The precipitated DNA was spun out and air-dried. It was then resuspended in 1 µl of hybridisation buffer, denatured at 85 °C for 5 minutes, and incubated at 37 °C for 15-30 minutes to block repetitive elements in the probe.

2.2.5 Metaphase spread preparation

For metaphase chromosome preparation from primary cells, 0.5 ml of peripheral blood from a volunteer was cultured for 72 hours in 10 ml RPMI-1640 with 10% FCS and 0.1 ml of reconstituted phytohaemagglutinin (PHA) (Life Technologies). These cells were then harvested as follows. Cultures were incubated with colcemid (Gibco) at a final concentration of 0.05 mg/ml for 30 minutes.

Cells were then centrifuged at 300 x *g*, resuspended in 0.075 M KCl, and incubated at 37 °C for 12 minutes. The cells were pelleted again and the KCl removed before suspending gently in 3:1 methanol:glacial acetic acid as fixative. The cells were pelleted and fixed twice more before being dropped onto glass slides. Slides were soaked in methanol and wiped clean prior to use. The quantity and quality of the metaphases were checked using phase microscopy. If the chromosomes were insufficiently spread, the evaporation of the fixative was slowed by drying the slide over a water-bath. The density of the cells was checked and adjustments made by the addition or removal of fixative. Slides were stored with desiccant at –20 °C and used within one month.

2.2.6 Fluorescence in-situ hybridisation on metaphase spreads

Slides with metaphase spreads were equilibrated to room temperature, and then artificially aged in a microwave oven. The preparations were treated with 100 µl Denaturation Buffer (70% deionised formamide, 2 x standard saline citrate, SSC) at 74 °C for 2 minutes and dehydrated through an ethanol series (70%, 95% and 100%). The pre-annealed probe (200 ng) was then applied to the slide under a coverslip and incubated over two nights in a moist chamber at 37 °C.

Standard post-hybridisation washes were 50% formamide, 2 x SSC (pH 7) at 42°C followed by 1 x SSC or 2 x SSC at 42 °C. Slides were rinsed with 4 x SSC, 0.05% Tween 20 (SSCT) and pre-incubated with SSCT plus 5% low fat dried milk (Marvel) (SSCTM). For detection of hybridised probes, all antibodies were diluted in SSCTM and 100 µl of each was applied for 30 minutes at 37°C. Biotin-labelled probes were detected with 5 mg/ml avidin-FITC DCS (Vector Labs) followed by 5 mg/ml biotinylated anti-avidin (Vector Labs) and a further round of avidin-FITC. Between incubation steps, the slides were rinsed three times in SSCT on a shaking platform at room temperature for 3 minutes. Finally, they were washed twice in PBS-A for 3 minutes and dehydrated in an ethanol series, 3 minutes each. Metaphase spreads were mounted in Citifluor AF1 antifade solution (Citifluor Ltd) containing 200 ng/ml DAPI (4'6'-diamidino-2-phenylindole) counterstain.

2.2.7 Immunofluorescence

For immunofluorescence, cells grown in chamber slides or attached to polylysine slides were fixed with 4% paraformaldehyde for 10 minutes, and permeabilised with 0.5% Triton-X in PBS-A for 20 minutes. For cells pulsed with BrdU, additional treatments of

0.1 M HCl for 10 minutes, and Denaturation Buffer for 2 minutes at 73 °C were applied. Slides were then blocked in SSCTM. Incubations with primary and fluorochrome-conjugated secondary antibodies were performed in SSCTM for 30 minutes each at 37 °C, with three washes in SSCT following each incubation. Slides were mounted in Citifluor AF1 with DAPI at a final concentration of 200 ng/ml, which also allowed counterstaining of the nucleus.

2.2.8 Combined immunofluorescence and FISH (ImmunoFISH)

For immunoFISH, cells were grown, fixed and permeabilised as previously described (Shiels *et al.*, 2001). This fixation technique was previously shown to preserve nuclear architecture at light microscopy resolutions (Kurz *et al.*, 1996). Essentially, cells grown or attached on slides were pre-extracted in cytoskeletal (CSK) buffer, containing 0.5% triton X-100 for 5 min on ice. Cells were then fixed in 4% formaldehyde for 10 min, and further permeabilised for 20 min with 0.5% triton-X in PBS. To facilitate detection of DNA sequences by FISH, optional nuclear permeabilisation steps were performed by repeated freeze-thawing in liquid nitrogen and/or treatment with 0.1 M HCl for 10 min. At this stage, cells could also be stored at 4 °C in 70% ethanol, and then equilibrated in 2 x SSC for 30 minutes prior to subsequent usage.

For visualisation of genomic loci by FISH, cells were denatured in Denaturation Buffer at 72 °C for 3 to 5 min and washed for 1 min in cold 2 x SSC before addition of denatured DNA probe. Each slide was then hybridised with 300 ng of denatured and biotin- and/or digoxigenin-labelled DNA probe under a 20x20 mm coverslip, at 37 °C overnight in a humid chamber. This was followed by washes at 42°C in 50% formamide, 2 x SSC for 3 x 5 min and in 2 x SSC for 3 x 5 min. Biotin-labelled DNA probes were detected using avidin-FITC streptavidin-alexa 546 (Molecular probes) or streptavidin-Cy5 (Cambio), and digoxigenin-labelled DNA probes were detected using mouse and sheep mouse anti-digoxigenin-FITC or –TR (Sigma and Boehringer Mannheim Corp.). Detection of nuclear proteins by immunofluorescence (as described above) was performed simultaneously with the FISH detection steps. Cells were counterstained in DAPI as above. Alternatively, WI-38 female diploid fibroblast cells were counterstained in propidium iodide (PI) at 1 μg/ml. The inactive X-chromosome was identified as a densely-stained Barr body.

2.2.9 RNA-FISH

The RNA-FISH procedure was adapted from Jolly *et al* (Jolly *et al.*, 1997). For these experiments, the biotinylated genomic probes for TAP/LMP, *HSPA5* and *COL1A1* were used at higher amounts of 600 ng to 900 ng per probe per slide. Cells grown on chamber slides were fixed and permeabilised as for DNA FISH. Ribonuclease (RNase)-free reagents and glassware were used in all steps where possible, and vanadyl ribonucleoside complex (New England Biolabs) was added to the permeabilisation and hybridisation buffers at a final concentration of 20 mM. This included the pre-fixation CSK buffer, post-fixation Triton-X/PBS-A and hybridisation buffer for the DNA probe. An additional step of treating cells with 3% hydrogen peroxide in PBS-A was performed to quench endogenous peroxidase activity. The slide denaturation step was omitted and the DNA probes were allowed to hybridise to RNA in the cells at 37 °C overnight.

The next day, slides were washed in Section 2.2.8, but with RNase-free reagents. Detection of biotin on the RNA-DNA hybrids was performed using the tyramide signal amplification (TSA) system (PerkinElmer Life Sciences). In summary, cells were blocked in TNB buffer (Tris-HCl, NaCl, BSA), for 30 min at room temperature. Cells were then incubated for 30 min at room temperature with streptavidin-horseradish peroxidase (HRP) (Dako) at 1:500 in TNB buffer, washed 3 times (5 min) in TNT buffer (Tris-HCl, NaCl, Tween-20), and incubated with the FITC Tyramide Working Solution (PerkinElmer). Three further washes were performed in TNT buffer.

Subsequent steps were performed to allow detection of genomic loci and immunofluorescence on the same slide. Cells were then treated with ribonuclease A (Sigma) at 200 U/ml and heat denatured as above (which also removes any peroxidase activity from the HRP previously used to deposit the tyramide signal). DNA FISH (using digoxigenin-labelled probes for the same gene loci) and immunofluorescence were performed as above.

2.2.10 Fluorescence and confocal microscopy

For visualisation of DNA probes on metaphase spreads, slides were examined with a Ziess Axioplan fluorescence microscope equipped with an automated filter wheel, Plan-Neofluar 100x oil immersion objective (Ziess), 10x eye objectives and a 0.5–2x Optivar (Ziess), giving between 1,000–2,000x magnification. A dual band pass filter (Vysis)

was used for simultaneous visualisation of FITC (green, probe signal) and DAPI (blue, chromosome banding). Images were captured using a cooled coupled device (CCD) camera (Photometrics) and image processing software (IPLabs).

For three-dimensional analysis of nuclei, image capture and analysis were as described previously (Shiels *et al.*, 2001). Essentially, images were captured as optical sections of 0.4 μm, using a Zeiss LSM 510 confocal laser scanning microscope equipped with a Plan Apo 63x/NA 1.4 objective. Three different wavelengths of lasers were used (488nm, 543nm and 633nm), which allowed the capture of up to three different fluorochromes. These were FITC or Alexa-488 (green); rhodamine, TR or Alexa-546 (red); and Cyanine-5 (Cy5) or Alexa-633 (far-red, but coloured by software to be blue). Sections were collected at 0.4 μm intervals through each nucleus using the LSM 510 imaging capture software. Images were captured as a series of tagged image files (TIFF), corresponding to each optical section.

2.3 Image analysis and statistical methods

2.3.1 Image processing and 3-dimensional reconstruction

For distance measurements, confocal image stacks were processed using the Image3D program. This is a UNIX-based programme written by Dr Suhail Islam and Prof Michael Sternberg (formerly Biomolecular Modelling Laboratory, Cancer Research UK). This software enables the analysis of the stacks of TIFF images, corresponding to the sections across a nucleus, generated by the confocal microscope (Figure 2.2.A-D). Using manually determined cut-off values of colour intensity for each colour channel (red, green and blue), the programme connects each 3D pixel (or voxel) with neighbouring voxels to construct triangles of colours. The triangles were further assembled into 3D bodies (Figure 2.2.C). The programme then produces 3D co-ordinates of centroid positions for each locus and PML body, allowing the Euclidean or vectorial distance between any two foci or objects to be calculated in μm (Figure 2.2.D). See Figure 2.1.E & F for an example of the reconstruction of a nucleus.

For each locus, the software then allows the distance to its nearest PML body, referred to as the minimum locus-PML distance (md-locus), to be calculated. The mean of the minimum locus-PML distance (mmd-locus) for the two alleles can then obtained for each nucleus. In addition, for each PML body in the nucleus, the minimum distance to

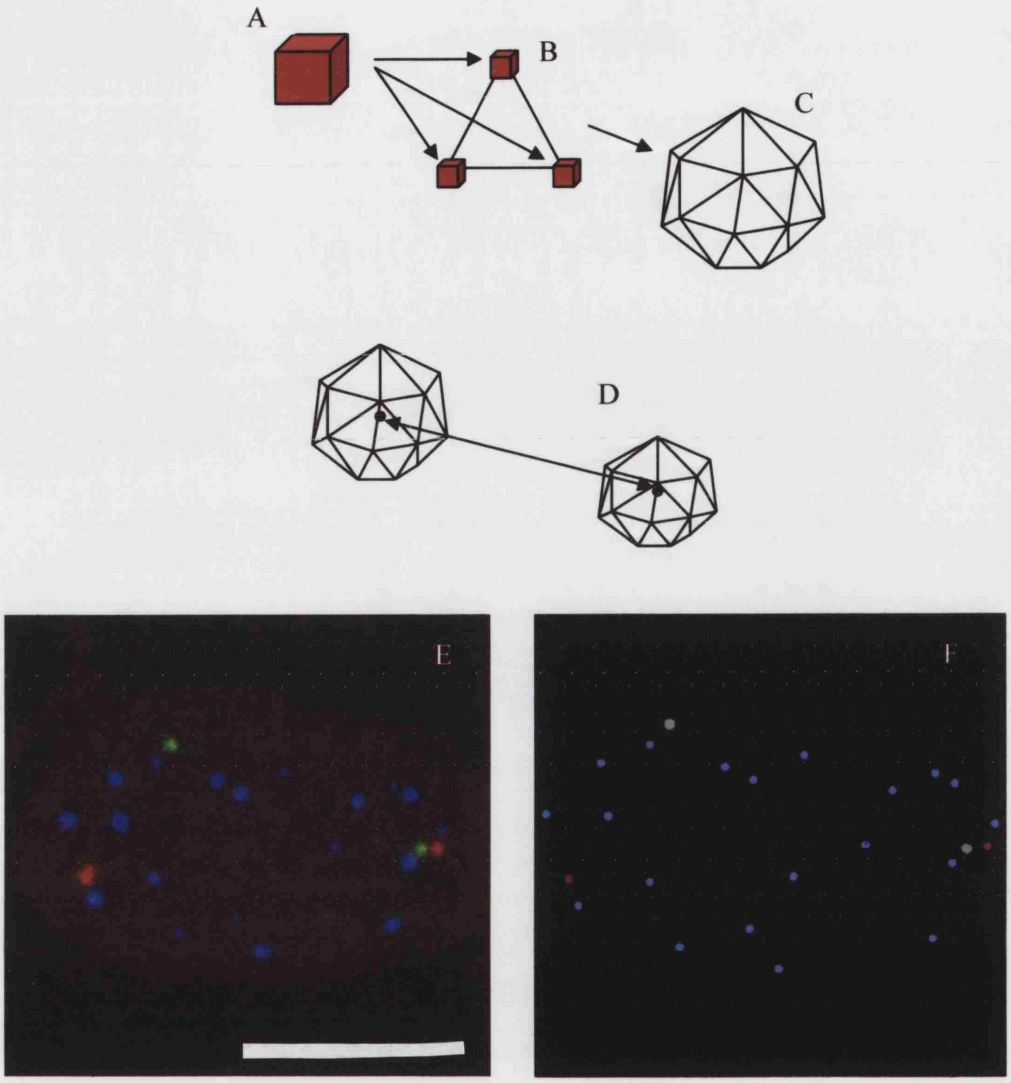

Figure 2.2. The reconstruction of 3-dimensional objects by the Image3D programme.

The software first analyses each 3-dimensional pixel (voxel) measuring 0.08μm x 0.08μm x 0.4μm (A), which has a minimum colour intensity. Neighbouring voxels with such cut-off values are analysed to determine if a triangular shape can be formed (B). Neighbouring triangles are then connected, and the programme determines whether a 3-d structure can be formed (C). The centroid of each 3D structure is determined giving a set of XYZ coordinates. The Euclidean distance between any two structures (having either the same or different colour) can then be vectored (D).

E: MRC-5 fibroblast cell nucleus, detected for PML (blue), the TAP/LMP locus (red), and the *ABC2* gene on chromosome 9 (green). F: Image of nuclear structures digitised into 3D construct. The white bar represents 5 micrometres.

its nearest neighbour PML body was measured, and the mean of all minimum PML-PML distances (mmd-PML) calculated for each nucleus (see Figure 2.3).

2.3.2 Statistical analysis based on distance measurements

At least 30 nuclei were captured per experiment and the md- and mmd-locus and mmd-PML calculated for this population. Where both alleles are considered equivalent, the mmd-locus (average of both alleles) was used. For studies where the two alleles of a genomic region were to be considered separately (e.g. the active/inactive copies of chromosome X genes, locus position within chromosome territories, and genes that had RNA transcripts detectable), the md-locus was used.

Where three-color experiments were used to compare directly the mmd-locus values for two loci in a population of cells, the values of mmd-$locus_1$ (e.g. green probe signal) and mmd-$locus_2$ (e.g. red probe signal) were obtained (see Figure 2.3). The PML body signal would be far-red, but coloured blue on the TIFF pictures. For statistical comparisons between the mmd for the two loci, the paired Student's t-test was performed using two-sided p-values, assuming the null hypothesis of equal means for the two loci. For single alleles where the two copies in a nucleus were to be compared directly (e.g. active/inactive X genes), the paired t-test on the md-$locus_1$ and md-$locus_2$ would be performed.

In some experiments, comparisons of mmd-locus between two different sets of cells were performed. Examples of these were interferon-treated versus untreated cells, cells in G0/1 phase vs. cells in S-phase. Here, $locus_1$ and $locus_2$ were measured from different cell populations, and an unpaired t-test was performed on the two sets of mmd-locus values under the assumption of equal variance. Where the mmd-PML distances are statistically different in the two sets of cells, the comparison was made between the differences. That is:

(mmd-$locus_1$)–(mmd-PML_1) vs. (mmd-$locus_2$)–(mmd-PML_2),

where 1 and 2 are from different cell populations.

When single alleles were analysed from different cell populations, md-locus and mmd-PML measurements were used. The difference in the association of PML bodies with two loci is said to be statistically significant if the p-value is less than 0.05, which corresponds to t scores of <-1.96 or >1.96.

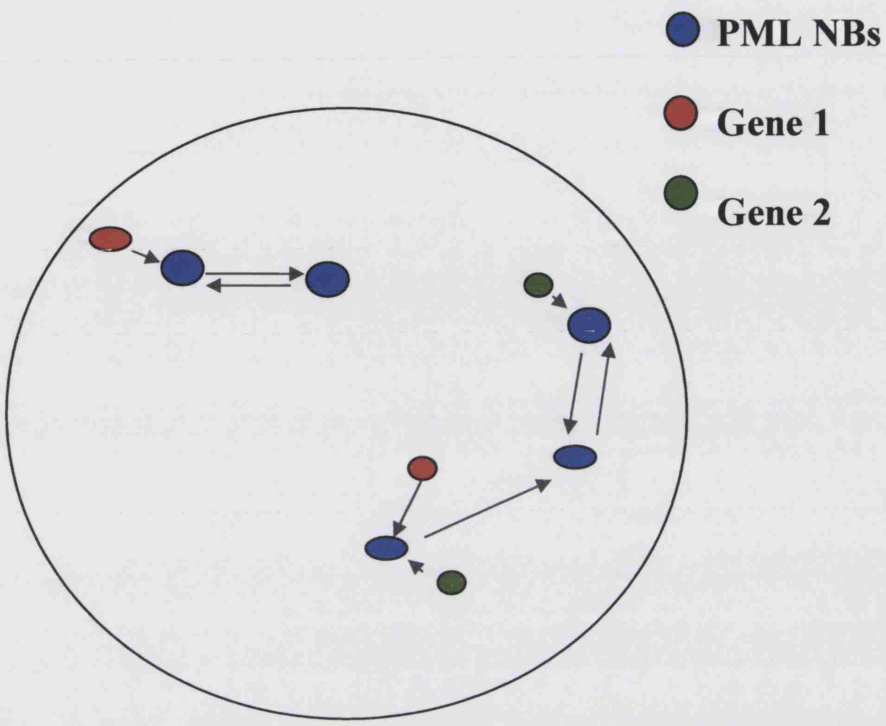

Figure 2.3. The statistical analysis of locus-PML associations by distance measurements.

The analysis of locus-PML distances for two different pairs of loci in a population of cells is analysed using the Student's t-test. For each nucleus, the following were calculated:

Red1 to nearest blue, Red2 to nearest blue, then mean red-blue (locus1-PML) distance.

Same for green-blue (locus2-PML) distance.

For n>30 nuclei, (red-blue distance) vs (green-blue distance), compared using the paired Student's t-test. $t < -1.96$ or > 1.96 = significant at $p<0.05$.

Genomic loci from different sets of nuclei are analysed by the unpaired t-test.

2.3.3 Other scoring criteria and statistical measurements

Where required, direct contact between PML bodies and other loci or structures was assessed by two independent observers examining a three-dimensional stack of images. Each locus or body was scored to be either physically touching a PML body or not. In the case of the positioning of genomic loci with respect to chromosome territories, loci were scored to be internal, peripheral or external to the chromosome territory edge. Percentage of loci in contact with a PML body (for genomic and transcript loci) or percentage of PML bodies in contact with other signals (for chromosome territories, replication or transcription foci, or nucleoli) were calculated as the mean of the two observations.

Statistical analysis of these non-parametric scores was performed using Chi-square tests to compare the scores for different populations of cells. Contingency tables (either 2 x 2 or 3 x 2) were used. A variation of the standard Chi-square test was used, the Chi-square test for trend.

2.4 Analysis of transcription

2.4.1 Total RNA isolation

RNA was extracted from cells using TRIzol reagent (Gibco), according to the manufacturer's protocol. This kit is based on phenol/chloroform extraction, in which DNA, RNA and protein are extracted into different phases of the mixture. MRC5 or CAKI-1/2 monolayer cells were lysed in 1 ml of TRIzol solution per 10 cm^2 of cells in incubation flasks. Cells were not washed before addition of TRIzol to reduce the possibility of mRNA degradation. After incubation for 5 minutes to permit the complete dissociation of nucleoprotein complexes, the RNA was separated from DNA and proteins by the addition of 0.2 ml of chloroform. The sealed tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for a further 2-3 minutes. Samples were centrifuged at 12,000 x *g* at 4 °C for 15 minutes. After centrifugation, the RNA remains exclusively in the colourless upper aqueous layer. This aqueous layer was transferred to a fresh tube and the RNA precipitated by mixing with 0.5 ml of isopropyl alcohol. The samples were incubated at room temperature for 10 minutes before centrifugation at 12,000 x *g* at 4 °C for 10 minutes. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube. After removing the supernatant, the RNA pellet was washed once in at least 1 ml of 75% ethanol. The

sample was vortexed before its final 12,000 x *g* centrifugation for 5 minutes. The air-dried RNA pellet was resuspended by incubation at 55-60 °C for 10 minutes in RNase free water or 0.5% SDS solution. The amount of RNA obtained was quantified by UV spectrometry, with one A260 OD unit being equivalent to 40 μg/ml of RNA.

2.4.2 Reverse transcription

One microgram of total RNA was then reverse transcribed using the First strand cDNA Synthesis kit (Amersham Pharmacia), using random hexa-deoxynucleotide triphosphate (dNTP) primers according to the manufacturer's instructions. RNA in 20 μl of RNAse free water was mixed with 11 μl of bulk first-strand reaction mix, 1 μl of primer and 1 μl of dithiothreitol (DTT) solution, and the sample incubated at 60 °C for 1hour. The reaction was inactivated at 95 °C for 5 minutes, and diluted to 5 μl with water. The cDNA was stored at –20 °C.

2.4.3 Polymerase chain reaction (PCR)

PCR was typically performed using approximately 10 pg plasmid template (for testing the FISH probes), or 100 ng cDNA (see above). cDNA made from 100 ng of total RNA was mixed with 1 μM of each primer (Appendix B.2) and the appropriate volumes of the Taq PCR Master Mix (Qiagen) and distilled water. The PCR reaction was performed for 30 cycles. Amplification was performed on a GradientCycler (MJ Lab), with a heated lid to prevent recondensation of the mixture. The following cycling conditions were used: 95 °C for 5min, then 30 cycles of 95 °C for 1min, annealing temperature Tm for 1min, 72 °C for 1min. This was followed by 72 °C for 5min after the final cycle.

PCR products were loaded onto a 1.5% agarose gels labelled with ethidium bromide (see Section 2.1.4). The PCR product bands were scanned using UV with a capture software (Labworks, UVP), which allowed the intensity of the bands to be quantified. Relative intensities were calculated using the intensity of the expressed gene β-actin (*ACTB*) as the reference (=1):

Ratio of gene to *ACTB* = (intensity of gene) / (intensity of *ACTB*)

To compare expression in different cell populations, for example after RNAi or ATRA treatments, band intensities were first normalised on the *ACTB* gene for the

corresponding cDNA sample. The relative intensities for the treated group and the control group were then calculated:

$$\text{Ratio of treated to control} = \frac{\text{Intensity of gene – intensity of ACTB (treated)}}{\text{Intensity of gene – intensity of } \textit{ACTB} \text{ (control)}}$$

2.3.4 Real-time quantitative reverse transcriptase (RT)-PCR

For real-time PCR, cDNA samples were prepared as above. Primers were designed using Primer Express (Applied Biosystems), to have an annealing temperature Tm of 60 °C, and were selected to extend over an intron, or across an exon-intron boundary if possible (Appendix B.3).

300nM of each primer pair was mixed with cDNA from 100ng of total RNA and the appropriate volumes of SYBR green PCR Master Mix (Applied Biosystems) and distilled water. The PCR reaction was performed on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). The cycle conditions are 50°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. DNA amplification was detected by SYBR fluorescence intensity for each cycle in the PCR machine. Signal intensities were analysed on SDS version 1.7 (Applied Biosystems), and the Calculated thresholds (Cts) were manually set for each sample well. Averages of Calculated thresholds were obtained for duplicated samples, and corrected against the *ACTB* reference. The *ACTB* reference was used for each cDNA sample, and for each PCR reaction. The difference in Cts between treated and control samples then allowed the relative expression of a gene to be quantified, as follows:

$$\text{Ratio of treated to control} = 2^{-[(\text{Ct-treated} - \text{Ct-ACTB}) - (\text{Ct-control} - \text{Ct-ACTB})]}$$

The expression of each mRNA was therefore expressed as the ratio (in log –2 scale) of a treated population versus the untreated one. Each experiment was repeated at least once more, with different populations of cells, to allow standard error of means (SEM) to be calculated.

2.5 Analysis of protein levels

2.5.1 Cell lysis and protein electrophoresis

For quantification of protein expression, whole cell lysates were obtained by adding 1 cell volume of 2 x Electrophoresis sample buffer to MRC5 cells treated with RNAi, or

PML transfection, and controls. The lysate was boiled for a further 5 minutes to fully denature the proteins, before analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Equal volumes of protein samples were loaded on Tris-Glycine gels (Invitrogen) containing a gradient of 4-20% acrylamide, together with visible rainbow molecular weight markers (Amersham). Gels were run vertically using Sample buffer, under denaturing and reducing conditions. Gels were run at 80V until the samples migrated out of the wells, then at 125 V for at least 90min.

2.5.2 Immunoblotting (Western blotting)

Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham) using a semi-dry transfer unit. The acrylamide gel, a nitrocellulose membrane or the same size, and 6 pieces of filter paper were wetted with Transfer buffer, and excess solution removed. The gel and membrane was sandwiched between the filter paper, and placed in the transfer unit, and the transfer was performed at 86 mA for 90 minutes. Efficiency of transfer was assessed by the presence of the rainbow marker in the membrane, and by the detection of residual bands on the gel following incubation in Coumassie blue solution (Simply Blue, Invitrogen).

PML and β-actin protein bands were detected with mouse monoclonal anti-PML (1:100, PG-M3, Santa Cruz) and anti-β-actin (1:2,000, Sigma) antibodies, using a WesternBreeze kit (Invitrogen). Essentially, membranes were pre-wetted in water, and blocked with the supplied Blocking solution for 30 minutes, before incubation with primary antibodies diluted in Blocking solution. Membranes were then washed 4 times in supplied Washing solution, and incubated with alkaline phosphatase (AP) conjugated anti-mouse antibody solution, before another 4 washes. Membranes were then incubated with the chemiluminescence reagent for 10 minutes. Chemiluminescent signals were detected by Hyperfilm-ECL photographic plates (Invitrogen). Protein bands were quantified using LabWorks (UVP). The relative quantification of PML protein was performed by normalising against the β-actin protein band, and comparison made between different populations of cells treated with RNAi or overexpression.

Chapter 3: The association of PML bodies with the genome

3.1 Introduction

As described in Chapter 1, PML bodies lie predominantly in the interchromosomal domain rather than within chromosome territories (Bridger *et al.*, 1998; Plehn-Dujowich *et al.*, 2000). In order to gain a better understanding of their function, efforts have been made to define the spatial distribution of PML bodies in more detail, especially with respect to other protein structures in the nucleus or to certain genomic regions. For example, PML bodies are often found adjacent to SC-35 domains (Ishov *et al.*, 1997; Smith *et al.*, 1999). In one cell line, a marked association is seen with Cajal bodies, with at least one PML body forming a duplex with a Cajal body in each cell (Grande *et al.*, 1996). While such associations may result from the fact that all these bodies lie within the confines of the ICD, they may also reflect a functional interaction of PML bodies with SC-35 domains or Cajal bodies. In contrast, PML bodies do not appear to associate with the nucleoli, except under specific cell treatments (in particular, proteasome inhibition) (Mattsson *et al.*, 2001).

There is also evidence that PML bodies are spatially associated with functional nuclear domains. PML bodies have been found to associate with transcription foci (as labelled with fluorouridine), and other indicators of transcription such as hypophosphorylated RNA pol IIa and CBP (see Section 1.3.7.1) (Kiesslich *et al.*, 2002; von Mikecz *et al.*, 2000). This includes viral transcription (Section 1.3.8.3). Another marker of transcriptionally active genomic regions, H3 acetylated histones, also appear to cluster around some PML bodies (Boisvert *et al.*, 2000).

3.1.1 Studies of PML-genome interactions

These studies are consistent with the large body of evidence suggesting that PML bodies are involved, directly or indirectly, in transcription (Section 1.3.7.1). Because of this, the association of PML bodies with specific genomic loci has been studied. Previous work in this laboratory showed that in primary human fibroblast nuclei PML bodies associate with the Major Histocompatibility Complex (MHC), a gene-rich region of chromosome 6 (Shiels *et al.*, 2001). A description of the MHC region is given later. The closest association was with the MHC class II region (see Figure 3.1), which includes the TAP/LMP (Transporter Associated with antigen Processing/Large Multifunctional Protease) locus containing the *LMP2/PSMB8*, *LMP7/PSMB9*, *TAP1*

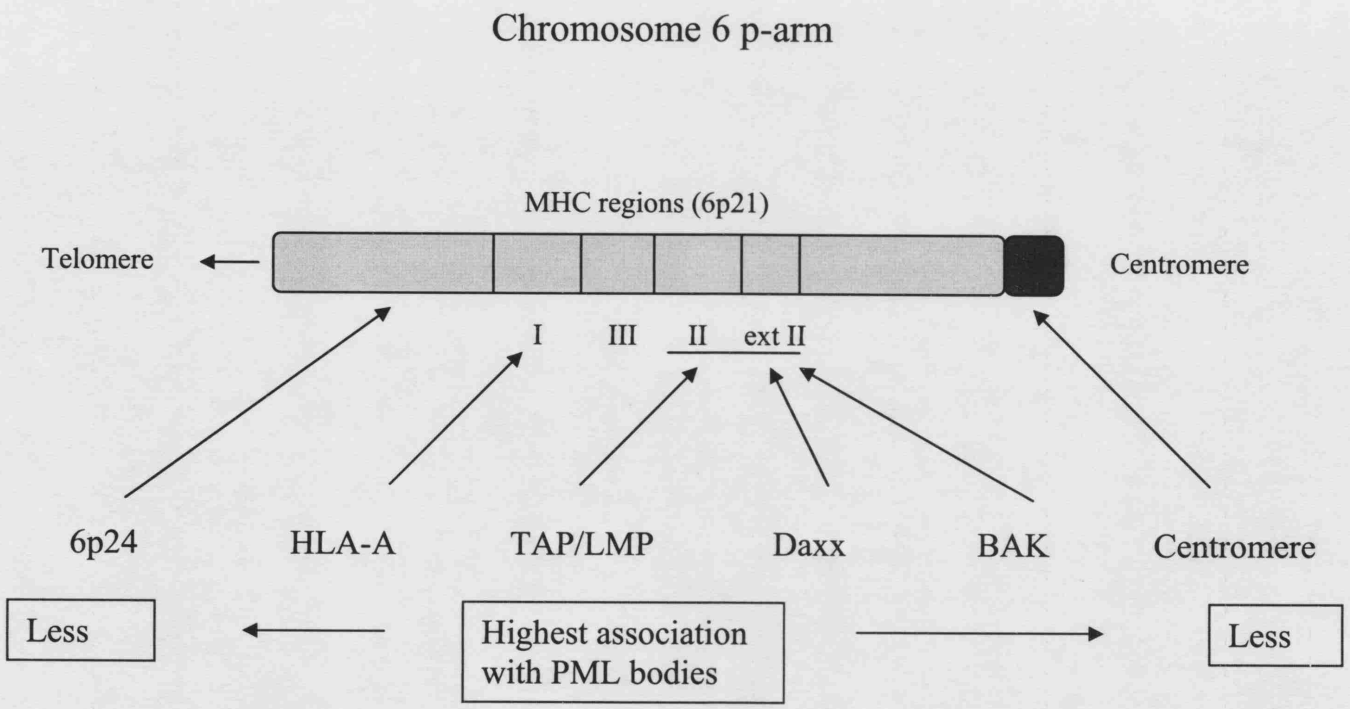

Figure 3.1. Diagram of the organisation of the MHC region on chromosome 6.
The MHC consists of the classes II, III and I, listed from the centromeric to telomeric end. The highest statistical association with PML bodies was seen in the class II region.

and *TAP2* genes, involved in MHC class I antigen presentation. Here, a PML body was found to be closely associated with the TAP/LMP locus in 42% of cells. To exclude association due to random factors, a novel statistical model was devised, as discussed in Chapter 2. Briefly, this was done by measuring the distance (in micrometres) from a genomic locus in a cell to its nearest PML body (termed the minimum distance, md), and obtaining a mean minimum distance (mmd) for a pair of alleles. The mmd values for a locus obtained from a population of cells form a binomial or normal distribution. This allows comparisons of the locus-PML association between different loci to be determined statistically in relative terms using the Student's t-test.

In contrast to the TAP/LMP locus, PML bodies were significantly less associated with the gene-poor 6p24 region and the chromosome 6 centromere (Shiels *et al.*, 2001). Surprisingly, PML bodies also showed a significantly weaker association with the MHC class I region. Furthermore, PML bodies associated less with the Epidermal Differentiation Complex (EDC) region on chromosome arm 1q, which is also gene-rich, but whose genes are largely unexpressed in fibroblasts. A specific lymphoblastoid cell line in which a region of the MHC class II (including the TAP/LMP locus) was deleted and reinserted as a YAC into chromosome 18 was also used. Here, the association of PML bodies with the remaining regions of the MHC (specifically the Daxx locus) remained as strong in terms of distance measurements, as the intact MHC. However, PML bodies acquired an association with the reinserted region of the MHC in chromosome 18 that was also comparable with the intact MHC. This was not noted for a native locus in the equivalent position on the other copy of chromosome 18. In was noted, however, that induction of MHC gene expression in fibroblasts by interferon-gamma (IFNγ) did not increase the association of PML bodies with this region. Thus, this study suggested that PML bodies may associate with a genomic region in a transcriptionally dependent manner, but not in acute response to transcription upregulation.

More recently, PML bodies were found to be associated with other regions. These studies were based only on the number of loci that were in direct contact with PML bodies. Williams *et al* looked at the association with PML bodies with the EDC, specifically in differentiating keratinocytes, compared with AHB lymphoblastoid cell line (Williams *et al.*, 2002). It was found that PML bodies associated with 22% of the EDC loci in keratinocytes, but only 12% in the lymphoblastoid cells. This difference did

not however reach statistical significance. In a more recent study using Jurkat cells, it was found that PML bodies associated with the *p53* gene (50% of the *p53* genes being in contact with a PML body), as compared with the *BCL2* gene (no *BCL2* gene was in contact) (Sun *et al.*, 2003). As with the previous paper, it was thought that this specific interaction was related to the transcriptional activity of the locus. However, it must be noted that PML bodies may also associate with other genomic regions, which is unlikely to be related to transcription. For example, PML bodies are found to associate with centromeres but only transiently in S/G2-phases (Everett *et al.*, 1999a).

Is the association of PML bodies with the MHC unique? If not, as suggested by Sun *et al* (Sun *et al.*, 2003), what functional elements of the MHC are important, and are there other regions that may likewise have similar associations? For this, a brief discourse of the characteristics of the MHC region is necessary.

3.1.2 Composition of the human major histocompatibility complex (MHC)

The human MHC (also known as the Human Leukocyte Antigen, HLA complex) is well characterised. In terms of physical and database resources, this region has been extensively mapped, and overlapping contigs are available covering the entire region. In addition, much is known about the sequence organisation and the transcriptional control of the classical MHC genes. Thus, both in terms of resources and our understanding of the region, the MHC is ideal for chromatin regulation studies.

The MHC locus was initially discovered by mapping the region encoding the classical HLA surface antigens, which are clinically important in tissue transplantation compatibility (reviewed in Browning and McMichael, 1996). In humans, the MHC maps to band 6p21.31 on the short arm of chromosome 6, and is divided into three classes based on the clusters of genes: class I, III and II (arranged from the telomeric to centromeric end) (Beck *et al.*, 1999). The entire region of about 3.6 megabases (Mb) has been sequenced and more than 200 genes and pseudogenes have been identified. The MHC is one of the most gene-rich regions in the human genome. Subsequently, the MHC was found to be remarkably conserved among the vertebrates, suggesting this clustering is important in evolution (see later).

Structural differences are marked between the different classes. For example, there is a distinct border between the class II and III regions. The classical class II region has been

shown to be an L2 isochore with 40.2% GC, while the class III region is an H3 isochore, with 53% GC. The GC content is more variable in the class I region, averaging 46% and is an H1 isochore (Beck *et al.*, 1999). The classical class II region also has an isochore boundary at its centromeric end, where it becomes the 'extended class II' region. The extended class II is ~51% GC (H2 isochore) with a sharp transition to class II (Stephens *et al.*, 1999). The isochore composition is also reflected in variations in gene density, with the 2 Mb class I region containing one gene per 14 kb, the 1 Mb class III region containing 1 per 11 kb, and the 800 kb class II region containing one gene per 40 kb (Beck *et al.*, 1999; Beck and Trowsdale, 1999; Shiina *et al.*, 1999). The structural differences between the different classes may also account for the fact that the MHC class II and III regions have been shown to replicate at different stages of the S-phase, with the class III region replicating earlier than class II (Tenzen *et al.*, 1997).

3.1.3 Functional aspects of the MHC

Functionally and clinically, the MHC plays an essential role in the immune response, and contains genes that process and present foreign and self antigens on cell surfaces. It is estimated that 40% of the expressed genes have immune-related functions (Beck *et al.*, 1999). The region contains genes that are constitutively expressed, as well as tissue-specific and inducible genes.

Both class I and II regions encode for antigen-presenting surface molecules. The class I region includes the *HLA-A,B,C* genes, termed the classical HLA genes, important for allograft rejection. The class I glycoprotein molecules are found on virtually all nucleated cells (reviewed in van Endert, 1999). These molecules bind short peptides derived from degraded cytoplasmic proteins (see later), and present them to CD8+ cytotoxic T-cells. These T-cells are alerted if the peptides presented on a cell are foreign, such as viral peptides, and they will mediate the destruction of the infected presenting cell.

The MHC class II region contains the genes *HLA -DP, -DQ* and *–DR*, which encode the classical class II antigen presenting molecules (Beck and Trowsdale, 1999). The class II molecules, like the class I, are cell surface glycoproteins. These molecules bind peptides derived from exogenous proteins, degraded by the endocytotic processing pathway. These are then presented to CD4+ T-helper cells. Expression of the class II molecules is

restricted to the surface of professional antigen-presenting cells, such as B-lymphocytes, dendritic cells, and macrophages. However, non-lymphoid cells can be induced to express class II molecules (see Chapter 4).

The MHC also encodes other components of the antigen-presentation machinery. Within the class II region is a cluster of genes involved in antigen presentation through HLA class I molecules: *LMP2 (PSMB8), LMP7 (PSMB9), TAP1* and *TAP2*. These four genes are tightly linked, with *TAP1* and *LMP2* sharing a bidirectional promoter (Wright *et al.*, 1995). The genes are co-ordinately expressed, and are upregulated by both type I and II interferons. The gene cluster is referred to in this thesis as the TAP/LMP locus.

In contrast to the class I and II regions, the functions of the class III genes are more heterogeneous (Beck *et al.*, 1999; Sugaya *et al.*, 1994), but encode immunologically important products. Examples include components of the complement cascade (e.g. C2, C4 and factor B), the interferon-inducible heat shock proteins (e.g. HSP70) and genes involved in the inflammatory response (e.g. TNFα andβ). It also codes for proteins proposed to be important in development (e.g. NOTCH4 and PBX2).

3.1.4 Gene clustering and the MHC

In addition to being one of the most gene-dense regions in the human genome, many of the genes contained in the MHC are structurally and functionally related. This is consistent with the recognised phenomenon of clustering of functionally related genes, which allows for coordinated expression (Trowsdale, 2002). This is thought to arise from gene duplication (see next Section). Alternatively, the relocation of a gene to the vicinity of another functionally related gene might confer a selective advantage. For these immune related genes, linkage also allows sequence exchange, leading to advantageous polymorphic alleles. Alternatively, close clustering also allows linkage disequilibrium, allowing inheritance of specific haplotypes (Jeffreys *et al.*, 2001).

Gene clusters occur throughout the genome. Another example of gene clustering is the homeobox (Hox) gene clusters, containing genes that are expressed during differentiation in a regulated manner (Zakany *et al.*, 2001). The cluster of immunoglobulin and KIR (encoding natural killer (NK) cell receptors) gene families also allows expression to be coordinated in a cell type specific manner (Wilson *et al.*, 2000).

3.1.5 The MHC and paralogous genes

Although the modern MHC appears to have formed during the emergence of the jawed vertebrates, it has been suggested that earlier organisms have a clustering of genes from which the MHC has evolved; this is termed the primordial MHC, proto-MHC or Ur-MHC (reviewed in Kasahara, 1999). With the evolution of the jawed vertebrates, two rounds of large-scale chromosomal duplication are thought to occur. As well as forming the MHC, these correspond with the development of the adaptive immune system (Kasahara, 1997). This en-bloc duplication also results in the development of four regions, containing many genes paralogous to the MHC. These are found on chromosome 1, 9 and 19 (Figure 3.2, Table 3.1). However, duplication or translocation of smaller regions may also give rise to paralogous genes in other regions.

Although duplicated from the MHC, differences in gene order and functions exist between the different regions. For example, duplication of the MHC does not occur for all genes, such that not all genes in the MHC have paralogues in the other regions (see Table 3.1 for examples of these). Only the minority, such as the NOTCH family of genes, has one copy in each of the paralogous regions. It may be that duplicated genes were subsequently lost or inactivated (Escriva *et al.*, 2002). The order of genes in the different chromosomes is also not preserved along the chromosomes. An example of this is chromosome 1, which has paralogous regions in the p and q arms, with both arms containing genes from all three classes of the MHC (Shiina *et al.*, 2001). Finally, paralogous genes are often noted for their divergent functions following duplication. For example, the *ABC2* gene on chromosome 9q34 does not appear to be involved in immune functions, although it is paralogous to the *TAP1/2* genes (Luciani *et al.*, 1994).

Despite these differences, the paralogous regions share common features. For example, 9q33-q34, 1p11-p32, 1q21-q25 and 19p13.1-p13.3 are all gene-rich regions.

3.1.6 The antigen presentation pathway for MHC class I

The processing of peptide antigens for presentation on MHC class I molecules is a complex process, which as mentioned, involves other proteins apart from the HLA genes.

Proteasomes are large protease complexes that are involved in degradation of ubiquitin-tagged proteins in the cytoplasm and nucleus (reviewed by Fruh and Yang, 1999). The

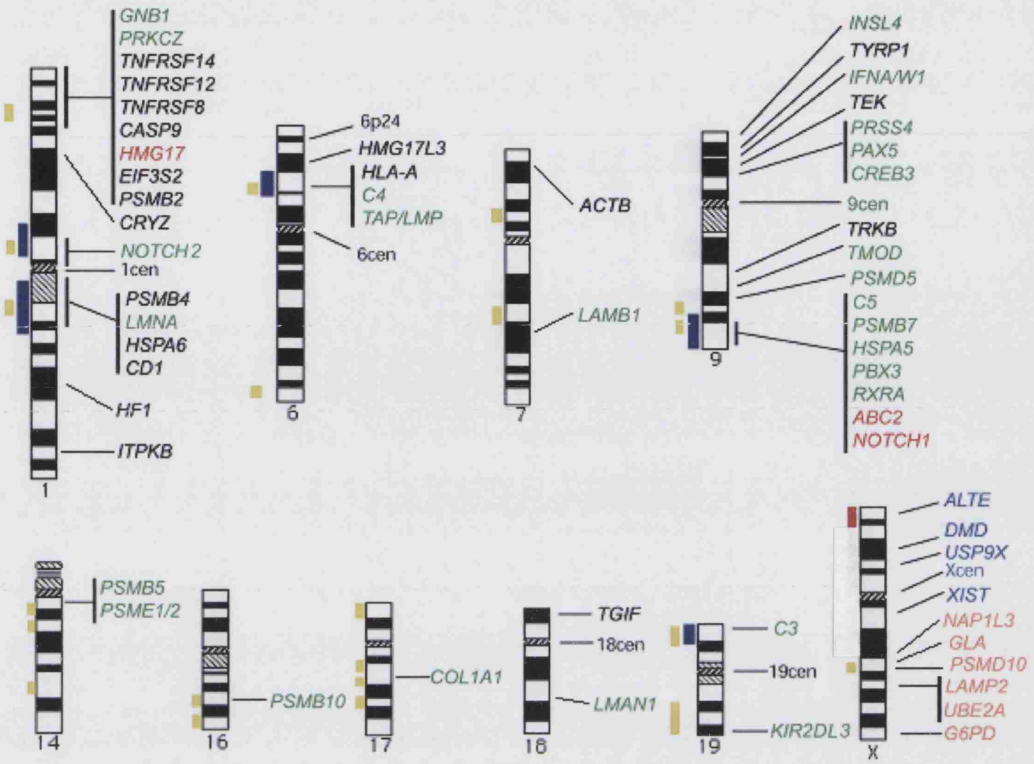

Figure 3.2. Genomic locations of loci analysed for association with PML bodies. Loci found to be more significantly associated with PML bodies than the TAP/LMP locus are shown in red. Loci which are as associated as the TAP/LMP locus are shown in green. Loci which are less significantly associated are shown in black. For the X-chromosome, loci on the active (Xa) and inactive (Xi) homologs were compared. Loci which were more significantly associated with PML bodies on the Xa- than the Xi-chromosome are shown in orange. Loci which are equally associated with PML bodies on both X-chromosomes are shown in blue. Dark blue bars against the chromosomes represent the MHC and regions paralogous to it. Yellow bars represent location of some of the genes encoding the proteasome subunits. Red bars represent location of pseudoautosomal regions on the X chromosome. (Refer to Table 3.1 & 3.2 and Appendix B.1 for cytogenetic locations of these loci.)

Table 3.1 The distribution of genes paralogous to the MHC.
Only genes in the MHC known to have paralogy are shown. Genes in the MHC are arranged from centromere to telomere. Shaded genes are tested in this thesis.

Chromosome 6	Chromosome 1	Chromosome 9	Chromosome 19
Class II			
SYNGAP	*NGAP* (1q25)		
RPL12L		*RPL12* (9q32-q33)	
BING1		*ZNF-X* (9q34)	
RGL2		*RALGDS* (9q34)	
B3GALT4	*B3GALT2* (1q31)		*AC005952* (19p13)
RPS18	*AL031733* (1q22-q23)		
RING1	*BAP1* (1q24-q25)		
RXRB	*RXRG* (1q22-q23)	*RXRA* (9q34.3)	
COL11A2	*COL11A1* (1p21)	*COL5A1* (9q34)	
RING3	*BRDT* (1p21-p22)	*RING3L* (9q34)	*HUNK1* (19p13)
LMP2/7		*PSMB7* (9q34)	
TAP		*ABC2* (9q34)	
Class III			
NOTCH4	*NOTCH2* (1p13-p11)	*NOTCH1* (9q34)	*NOTCH3* (19p13)
PBX2	*PBX1* (1q23)	*PBX3* (9q33-q34)	
LPAATA		*LPAATB* (9q34.3)	
PPT2	*PPT1* (1p32)		
CREBL1	*ATF6* (1q21)		
TNXA/B2	*TNR* (1q24)	*HXB* (9q33)	
C4B/A		*C5* (9q34.1)	*C3* (19p13)
NG35		*ZNF-X* (9q34)	*ZNF91* (19p13)
G9A	*KIAA0067* (1q21)		
NG22			*AF070636* (19p13)
HSPA1B/A/L	*HSPA6/7* (1q21)	*HSPA5* (9q34)	
CLIC1	*P64H1* (1p31-p33)	*CLIC3* (9q34)	
BAT2	*AL021579* (1q23-q24)	*KIAA0515* (9q34)	
AIF1	*AIF1*-like (1p33-p34)		
TNFSF3/B/F2/F1	*TNFSF4/6* (1q25,q23)	*TNFSFB* (9q33)	*TNFSF7/9* (19p13)
BAT1			*U90426* (19p12)
Class I			
MICB/A	*CD1* (1q21-q23)		
HLA-A/B/C/D/E/F			
POU5F1	*POU3F1* (1p34.1)		
DDR	*NTRK1* (1q21-q22)	*NTRK2* (9q22.1)	
TUBB		*TUBB2* (9q34)	
KIAA0170	*MSA* (1q25)		
GABBR1		*GABBR2* (9q21.3)	
OLFR2		*OLFR3* (9q21,q34)	*OLFR* (19p13.1)
Histone cluster	Histone cluster (1q21)		
HMG17L3	*HMG17* (1q36)		

proteasome consists of a cylindrical 20S catalytic core (consisting of PSM-A and B subunits) capped by two 19S ATPase complexes (containing PSM-C and –D subunits). Upon IFNγ stimulation, the subunits PSMB5, PSMB6 and PSMB7, are replaced by PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL-1) (Griffin *et al.*, 1998). Other subunits PSME1 (PA28α) and PSME2 (β) are also upregulated by IFNγ to form the PA28 proteasome regulator, which replaces the ATPase caps (Tanahashi *et al.*, 1997). This structure forms the immunoproteasome, a key component in the breakdown of foreign proteins for antigen presentation. *TAP* encodes a transporter associated with antigen processing, which delivers the resulting peptides into the lumen of the endoplasmic reticulum (Gubler *et al.*, 1998). In the lumen, the peptides co-operate with newly synthesised β2-microglobulin to complex with class I molecules, forming the class I/peptide heterodimer present on virtually every nucleated cell.

It is thought that the *PSMB7* gene at chromosome band 9q34 arises from duplication of the LMP genes in the MHC class II region. In fact, several other proteasome subunits reside in paralogous regions or in close proximity to each other, such as *PSMB2, PSMB4* and *PSMD4* on chromosome 1 and *PSMB5, PSME1* and *E2* on chromosome 14 (McCusker *et al.*, 1997). See Figure 3.2 and Table 3.2 for the locations of these genes.

We can therefore see that the MHC has many properties that may provide the basis for its association with PML bodies:

1. The MHC is one of the most gene-rich regions in the human genome.
2. It contains a large number of genes that are functionally related, especially with regard to the immune system and antigen presentation.
3. Many genes within the MHC are clustered and transcriptionally coordinated.

However, it is also noted that the genome contains several other regions that are functionally, or structurally related to the MHC, such as the paralogous genes that have been mentioned.

This chapter of my thesis is therefore based on the study of PML-genome associations, to extend the work previously done in this laboratory showing a spatial association of PML bodies with the MHC. My initial research was to validate the statistical model that was used. In particular, the relevance of the mmd measure with regard to functional association would be examined. Following this, I determined whether different genomic regions are likewise associated with PML bodies.

Table 3.2. Genomic locations of genes encoding proteasome subunits.
Locations are obtained from the NCBI LocusLink website and www.emsembl.com. The shaded genes are tested in this thesis.

Gene	Location	Gene	Location
α-subunit (20S)		ATPase subunit (19S)	
PSMA1	11q15.1	*PSMC1*	19p13.3
PSMA2	6q27	*PSMC2*	7q22.1-q22.3
PSMA3	14q23	*PSMC3*	11p11.2
PSMA4	15q24.1	*PSMC4*	19q13.11-q13.13
PSMA5	1p13	*PSMC5*	17q23.1-q23.3
PSMA6	14q13	*PSMC6*	12q15
PSMA7	20q13.3		
		Non-ATPase subunit (19S)	
β-subunit (20S)		PSMD1	2p37.1-q37.2
PSMB1	7p12-13	*PSMD2*	3q27.3
PSMB2	1p34.2	*PSMD3*	17q21.2
PSMB3	17q12	*PSMD4*	1q21.3
PSMB4	1q21	*PSMD5*	9q34
PSMB5	14q11.2	*PSMD6*	?
PSMB6	17p13	*PSMD7*	16q23-q24
PSMB7	9q34.11-q34.12	*PSMD8*	19q13.13
PSMB8	6p21	*PSMD9*	12q24.31-q24.32
PSMB9	6p21	*PSMD10*	Xq22
PSMB10	16q22.1	*PSMD11*	17q12
		PSMD12	17q24.3
		PSMD13	11p15.5
		PSMD14	2q24.3
		PA28 subunit (11S)	
		PSME1	14q11.2
		PSME2	14q11.2
		PSME3	17q21.32-q21.33

3.2 Results

3.2.1 Assessment of locus-PML associations using distance measurements

To determine whether PML bodies associate with other genomic regions to the same statistical significance (or degree) as the MHC class II region, a total of 54 loci from chromosomes 1, 6, 7, 9, 14, 16, 17, 18 and 19 were examined. These included gene-encoding loci as well as centromeres. The selection of the different loci was based on various factors. Many loci were chosen from chromosomes 1 and 9, since these chromosomes contain large regions that are paralogous to the MHC, and they also have variable gene densities along their lengths. Several loci from chromosomes 18 and 19 were chosen as these two chromosomes are of similar sizes, but vary markedly in their overall gene density. The selection of other loci was based on their functional significance, and will be described in detail in later sections.

All these loci were tested together with the TAP/LMP locus in the MHC class II region, by three-colour immunoFISH experiments to allow the simultaneous detection of DNA probe signals and PML bodies. The TAP/LMP locus was chosen as a reference, as Shiels *et al* previously found that PML bodies were most associated closely with this region (Shiels *et al.*, 2001). The mmd distances for all loci were therefore compared by paired t-tests against the TAP/LMP locus (see Chapter 2, Section 2.3.1-2 for the method used). Full results are presented in Appendix C.1 and summarised in Figure 3.2 (the loci studied along chromosome X will be discussed later in Section 3.2.3.4). In particular, PML bodies were found statistically to be more closely associated with the *HMG17* gene on chromosome 1, and the *ABC2*and *NOTCH1*genes on chromosome 9, than with TAP/LMP. PML bodies were found to be as closely associated with several loci as TAP/LMP, and less closely associated with other loci.

Several questions arose from this large-scale analysis. Firstly, was the correct analytical procedure used? Secondly, what is the biological basis of the variation found for the association between PML bodies and different loci? Thirdly, what do these findings tell us about overall nuclear organisation? These questions are addressed below.

3.2.2 Evaluation of the statistical method

The first question asked above was related to the relevance of the mmd measurements and the statistical method of comparing different genomic loci. This statistical method

was devised in our laboratory in collaboration with Drs Peter Sasieni, Paul Freemont and Michael Sternberg, as it was thought to overcome the subjective nature of visually counting direct contact between PML bodies and genomic loci. The main drawbacks however, are that mmd comparisons are not intuitive or widely used. This section therefore attempts to validate the method used.

Another comment regarding this statistical method is that such mmd comparisons are relative (that it, PML bodies are determined statistically to be more associated with one loci compared with another, but it is impossible to determine at which distance a mmd measurement is functionally relevant). This last observation is inherent in the statistical model, and cannot be fully addressed except by examining the characteristics of the loci involved. For example, most centromeric loci were among the least associated with PML bodies. However, it must be noted that the associations of different loci with PML bodies likely form a spectrum or continuous scale, rather than discrete categories, which I have artificially formed, by comparing different loci to the TAP/LMP locus.

3.2.2.1 Comparison of locus-PML association using distance measurements and direct contact scores

Although the experiments described here and in Shiels *et al* use mmd distance measurements, many other studies on gene associations with nuclear structures such as Cajal bodies and SC-35 domains are based on visual assessment of direct contact (Shopland *et al.*, 2001; Smith *et al.*, 1999; Xing *et al.*, 1995). I therefore wished to determine, firstly, whether the two different methods correlate with each other and whether the evaluation by distance measurements is valid. Secondly, I wished to determine whether the method of distance measurements is more robust than assessment of direct contact for determining locus-PML associations.

Thus, for each of the 54 loci studied, three scores of association were made: the percentage of loci in direct contact with a PML body, the percentage of loci within 1μm of the nearest PML body, and the mmd-locus measurements. For visual scoring of contact between PML bodies and other loci, each cell was examined as a three-dimensional stack of images by two independent observers. Each locus was scored to be either physically touching a PML body, or not. The percentage of loci in contact with a PML body was calculated as the mean of the counts from the two observers. As expected, and in agreement with others who have performed similar comparisons (Roix

et al., 2003), all three scores were found to correlate closely with each other (Figure 3.3). This suggests that mmd measurements can at least be used in place of direct contact scores.

However, I also noted that for loci that had different statistical associations with PML bodies, this was not due to a subset of signals in contact with PML bodies affecting the mmd scores. Rather, the frequency distributions of locus-PML distances for the two loci were different. A comparison of *PSMB4* and TAP/LMP is given here as an example, since PML bodies were found by mmd distance measurements to be less significantly associated with *PSMB4* than TAP/LMP region. The histograms of the minimal distances of *PSMB4* and TAP/LMP to the closest PML body are clearly seen to be shifted relative to each other (Figure 3.4).

I also found that the differences in mmd association of each locus with PML bodies (when compared with the TAP/LMP locus) persisted after scores for alleles that were less than 1 μm from PML bodies were removed from the data set (see Appendix C.2). Using *PSMB4* again as an example, in comparison with TAP/LMP, after scores for alleles that were less than 1μm from PML bodies were removed from the data set, the unpaired t-test revealed that *PSMB4* remained less significantly associated than TAP/LMP. Other loci, representing a spectrum of different degrees of association with PML bodies, *NOTCH1*, *IFNA1*, *NOTCH2*, *RXRA*, *TGIF*, and *TRKB*, gave similar results. These findings suggest that the association of PML bodies with certain loci does not require direct contact, but is based on the overall distribution of the mmd values. That is, some loci tend to be located closer to PML bodies, compared with others.

I also found, using mmd measurements, that PML bodies tended to be least associated with all centromeres tested, except the chromosome 9 centromere. However, due to the large FISH signals of centromeres, a high proportion of signals from centromeres were visually scored to be in contact with PML bodies. This led me to believe that the direct visual scoring system is more subjective and prone to artefacts, as the intensities and sizes of FISH probe signals can vary under different experimental conditions. I therefore felt that statistical measurements based on distances, and corrected for PML-PML distances, are a more objective method to assessing PML-locus association. This is especially so, because in the constraints of the nuclear space, random associations assessed by direct contact will always be an issue.

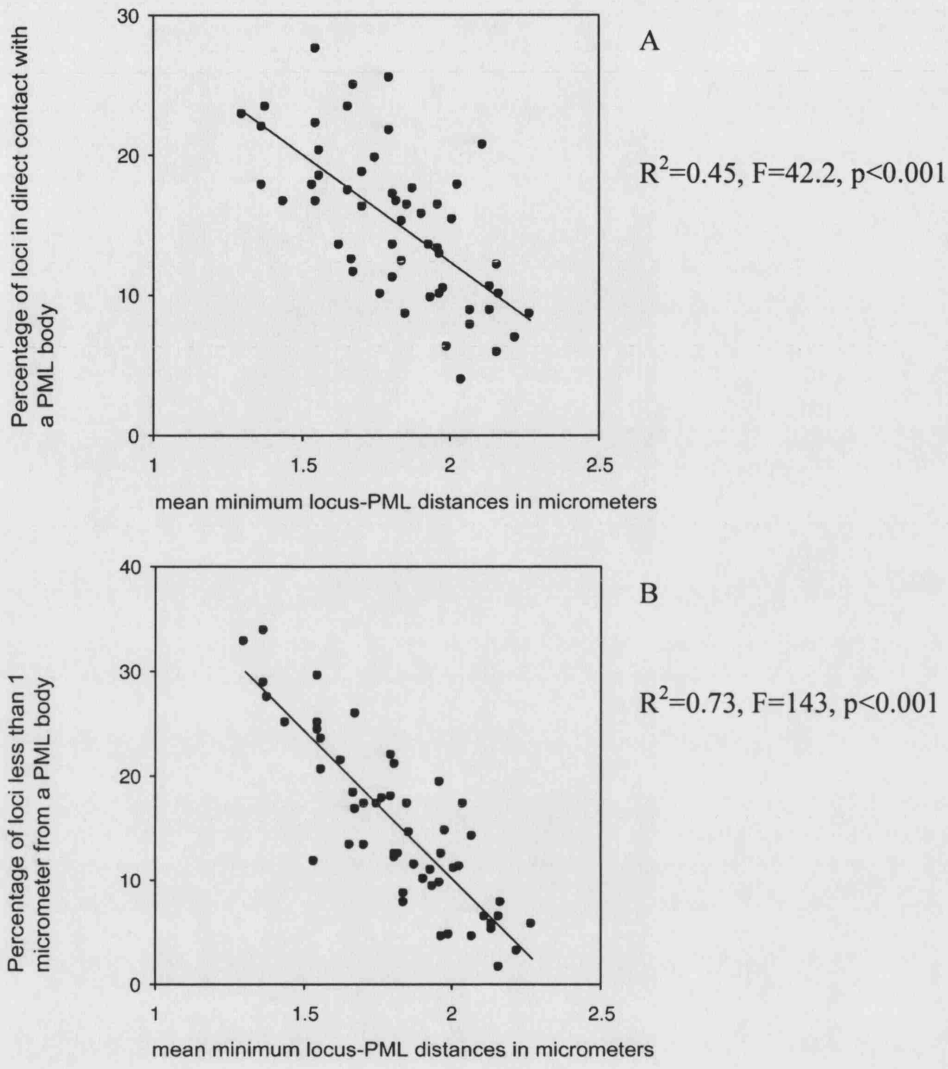

Figure 3.3. Linear regression graphs comparing the different measurements of locus-PML association.

The locus-PML association as represented by mmd-locus in micrometers is compared with the association as measured by the percentage of loci in direct contact with PML bodies assessed visually (A), and the percentage of loci less than 1μm from the nearest PML body (B). Each bullet represents a locus. Each graph displays the best line fit for the regression.

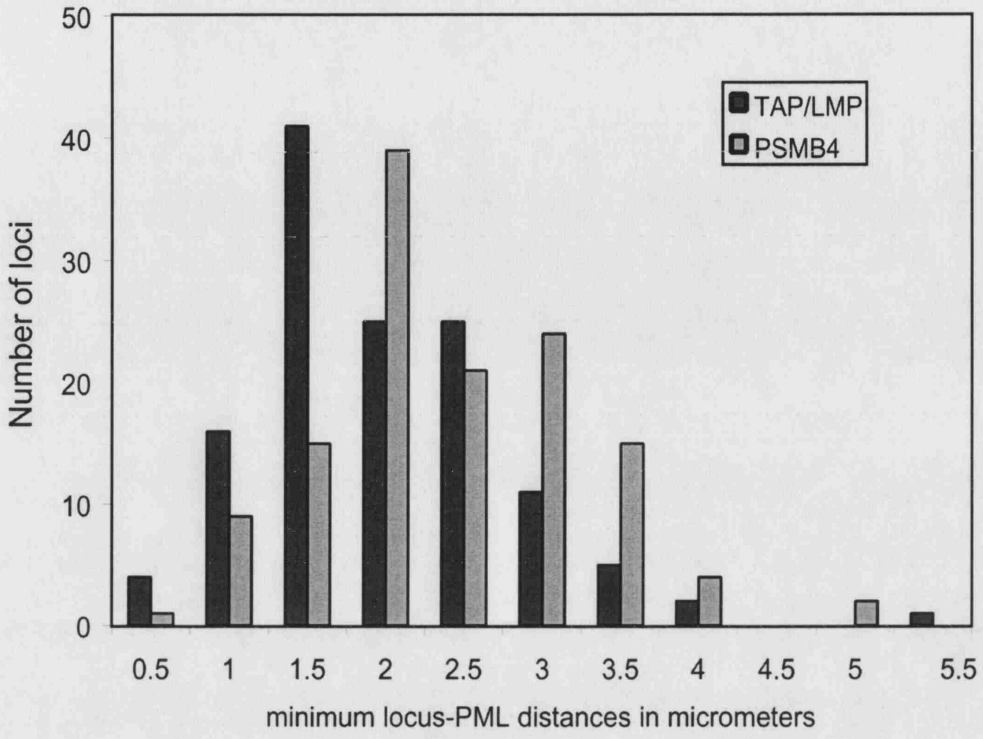

Figure 3.4. Histogram representing the minimal locus-PML distances for TAP/LMP and *PSMB4*.

The frequency distribution of minimum locus-PML distances for the TAP/LMP and *PSMB4* loci, which were found to be statistically different in their association with PML bodies using the Student's t-test. This statistical significance was retained for these loci following exclusion of measurements of less than 1μm.

3.2.2.2 Association of PML bodies with genes in close proximity on a chromosome

Next, I wished to determine whether the association of PML bodies with a locus is related to the association of other closely positioned genes on the same chromosome. I first examined whether this was true in the MHC region. For different pairs of genes within or near the MHC and separated by variable distances, I studied whether either gene was closer to the nearest PML body in three-colour experiments. Comparisons were made by mmd distance measurements and by direct visual assessment.

The TAP/LMP locus was first compared with the *C4B* gene in the class III region. These loci are separated by ~800 kb, and PML bodies associate to the same degree with both genes using the mmd method (see Appendix C.1). Comparison of the distances of the TAP/LMP locus and the *C4B* gene to the nearest PML body in each nucleus in turn showed that neither locus is consistently closer to a PML body than the other (Figure 3.5.A, Table 3.3.A). Visual assessment then showed that 9.6% of *C4B* signals and 8.6% of TAP/LMP signals were in direct contact with a PML body. Furthermore, 57% of *C4B* signals were closer to a PML body than their linked *TAP/LMP* signals, while 43% of *TAP/LMP* signals were closer. The TAP/LMP locus was next compared with the *HLA-A* gene, which had been found using the mmd method to be less significantly associated with PML bodies than the TAP/LMP locus. These loci are separated by ~2.3 Mb. Here, 60% of TAP/LMP signals were closer to a PML body than their linked *HLA-A* signals. The TAP/LMP locus was then compared with the *HMG17L3* gene on chromosome band 6p22, which was also assessed using the mmd method to be less significantly associated with PML bodies than TAP/LMP. These loci are separated by ~6.2 Mb. In this experiment, 67% of TAP/LMP signals were closer to a PML body than their linked *HMG17L3* signals.

Taken together, the above findings suggest that the observed associations between PML bodies and the different loci do not depend on one specific locus, such as TAP/LMP, 'pulling' a second locus towards a PML body, such that the second locus then becomes associated by proxy. If that was the case, then we would see one locus consistently closer to a PML body, than another locus along the chromosome.

Other loci on chromosomes 1 and 9 were also examined. The *ABC2* and *PSMB7* genes in band 9q34 are separated by 16 Mb. PML bodies show strong associations with both genes by mmd measurements (PML bodies were more significantly associated with the

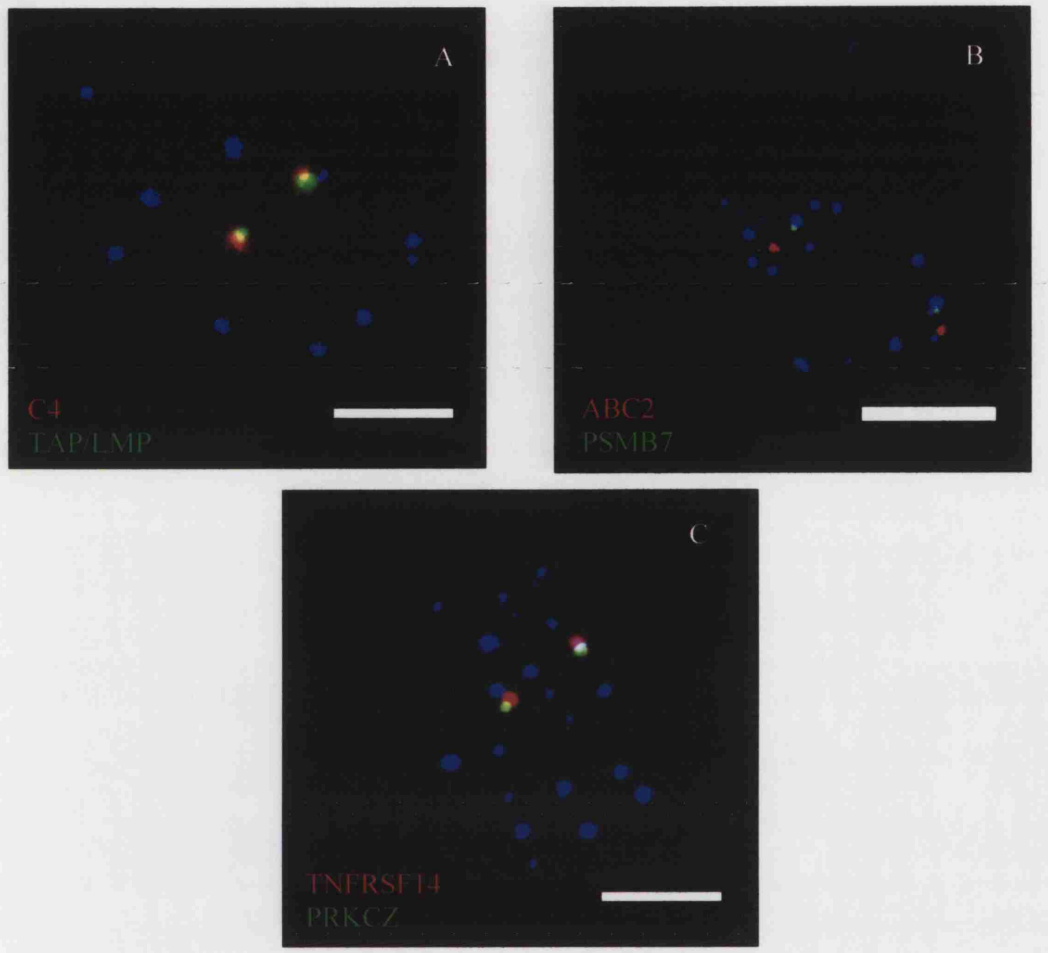

Figure 3.5. The association of PML bodies with loci in close proximity with each other.

A: MRC5 cell nuclei showing the *C4* gene in the MHC class III region (red), the TAP/LMP locus in the MHC class II region (green) and PML bodies (blue). The solid line represents 5 micrometers. Analysis of the *C4* and TAP/LMP loci (about 800kb apart on chromosome 6) together showed that neither locus was consistently more often in direct contact with a PML body than the other.

B: MRC5 cell nucleus showing the *PSMB7* gene (green), the *ABC2*gene (red) and PML bodies (blue). The two genes, 3Mb apart along chromosome 9, showed occasional contact with different PML bodies.

C: MRC5 cell nucleus showing the *PRKCZ* gene (green), the *TNFRSF14* gene (red), and PML bodies (blue). The *PRKCZ* and *TNFRSF14* genes, which are about 500kb apart on chromosome 1, showed statistically different levels of association with PML bodies.

The solid line represents 5 micrometres.

Table 3.3. Analysis of locus-PML association by direct contract for loci along chromosome 6

A

Locus	Map distance to TAP/LMP (Mb)	Physical distance (μm)	Cells (n)	% Locus closer to PML body	% TAP/LMP closer to PML body
C4	0.8	0.43	32	57%	43%
HLA-A	2.0	1.17	28	40%	60%
HMG17L3	3.5	1.40	32	23%	77%

B

Locus	Map distance between loci (Mb)	Cells (n)	% *PSMB7* closer to PML body	% *ABC2* closer to PML body	% of loci pairs closer to different PML bodies
PSMB7	16.0	41	44%	56%	56%
ABC2					

C

Locus	Map distance between loci (Mb)	Cells (n)	% *PRKCZ* closer to PML body	% *TNFRSF14* closer to PML body
PRKCZ	0.5	32	84%	16%
TNFRSF14				

ABC2 gene than the TAP/LMP region, while the association with *PSMB7* gene was statistically the same as TAP/LMP). Here, different PML bodies were found to associate with *ABC2* and *PSMB7* (from the same chromosome) in 56% of chromosomes analysed, suggesting that PML bodies associate independently with loci separated by several megabases on the same chromosome (Figure 3.5.B, Table 3.3.B).

The genes *TNFRSF14* and *PRKCZ* in chromosome band 1p36 are separated by 500 kb. PML bodies were found by mmd measurements to have the same degree of association with *PRKCZ* as with TAP/LMP, while being less associated with *TNFRSF14*. A direct comparison by three-colour analysis using three-colour experiments, detecting both loci together confirmed that PML bodies were more significantly associated with *PRKCZ* than *TNFRSF14* (mmd-*PRKCZ*=2.01 μm, mmd-*TNFRSF14*=2.15 μm, t-score=4.84, $p<0.01$). This pair of genes was the closest in terms of genetic distances to have different degrees of associations with PML bodies (Figure 3.5.C, Table 3.3.C). Visual assessment showed that 86% of *PRKCZ* signals were closer to a PML body than their linked *TNFRSF14* signals.

3.2.3 Association of PML bodies with MHC-related genes

3.2.3.1 Association of PML bodies with paralogous genes

As mentioned, several features of the MHC distinguish it from other genomic regions. I therefore reviewed the findings in Section 3.2.1 in light of the relationships of the genes with the MHC. A survey of the regions on chromosomes 1 and 9 that are paralogous to the MHC, showed that PML bodies were as, or more, significantly associated with all seven genes tested from band 9q34 as TAP/LMP in the MHC. On the other hand, PML bodies were as significantly associated with only one of four genes tested from band 1q21-q23, as compared with TAP/LMP. PML bodies were not as significantly associated with the other three genes here.

Since the experiments described in Section 3.2.1.3 suggest that PML bodies associate independently with individual genes within a genomic region, a more detailed examination of these paralogous regions is appropriate (see Figure 3.6). As described above, chromosome band 9q34 is gene-rich and has a large number of genes that are paralogous to the MHC class II and III genes (Kasahara, 1999). Of particular interest are the genes for ATP-Binding Cassette-2 (*ABC2*) and Proteasome subunit B7 (*PSMB7*),

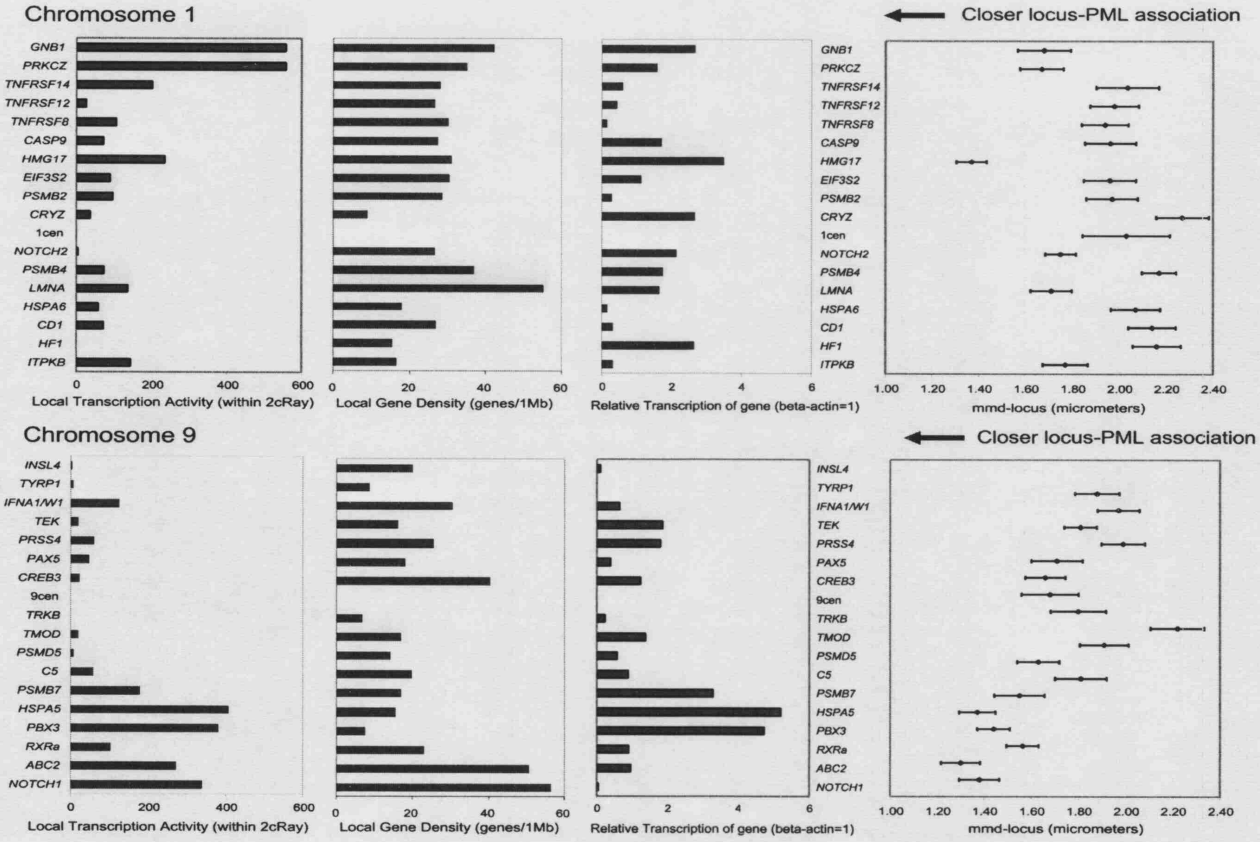

Figure 3.6. Comparison of locus-PML associations for genes on chromosomes 1 and 9 in relation to the local transcription activity, local gene density and individual gene transcription.

Local transcription activity was calculated for +/- 1 cR around each locus, local gene density was calculated for +/- 500 kb around each locus, and individual gene activity was determined relative to transcription of the β-actin gene. Locus-PML association is then represented by mmd-locus in micrometers with error bars showing 1 standard error.

which are paralogous to the *TAP1 & -2* and *LMP2/PSMB9 & LMP7/SPMB8* in the MHC, respectively. PML bodies were as or more significantly associated with all the genes in this region (*NOTCH1*, *ABC2*, *RXRA*, *PSMB7*, *HSPA5*, *PBX3* and *C5*) as TAP/LMP. PML bodies were also closer to the *ABC2* and *NOTCH1* genes on average than to the TAP/LMP loci.

To determine whether this result was specific to the 9q34 region, other genes along chromosome 9 were examined. The *TRKB* (*NTRK2*) gene is paralogous to *NTRK4* in MHC class III, but lies in the gene-poor 9p22 band, and is expressed only in neurally derived cells. PML bodies were less significantly associated with this gene than TAP/LMP. As a comparison, several non-paralogous genes were selected along chromosome 9. The *CREB3*, *PAX5*, *IFNA/W1*, *PRSS4* and *TMOD* genes, which are present in more gene-dense and GC rich regions of chromosome 9, showed similar locus-PML associations to TAP/LMP. In contrast, PML bodies were less associated with *TEK*, *ISNL4* and *TYRP1* (lying in relatively less gene-dense regions) than the TAP/LMP locus. Interestingly, *IFNA/W1* is positioned in a cluster of genes encoding other interferon proteins, and is known to be induced by interferon via a positive feedback loop.

The 1p12-21 and 1q21-23 regions also have a number of genes that are paralogous to the MHC, including *NOTCH2*, *CD1* and *HSPA6*. PML bodies showed similar associations with the *NOTCH2* gene in band 1p12 as TAP/LMP, but were not as significantly associated with *CD1* and *HSPA6* in 1p21-23. The 1q21-23 region is also gene-rich, and contains the EDC, which shows a lower degree of PML body association than the TAP/LMP locus, at least for the MRC5 cell line (Shiels *et al.*, 2001). PML bodies are likewise not significantly associated with many of the genes here, as compared with the TAP/LMP locus. The *PSMB4* gene is located here and as described in Section 3.2.2.1, is less significantly associated with PML bodies than TAP/LMP. In contrast, PML bodies were as associated with the lamin A/C (*LMNA*) gene as TAP/LMP.

To explore further the relationship between paralogy and association with PML bodies, 1p34-36 was also examined. This region is gene rich, but is not known to be a major paralogous region to the MHC (although several paralogous genes are located in this vicinity). Here, PML bodies were at least as significantly associated with the *GNB1*,

PRKCZ and *HMG17* genes as the TAP/LMP locus, but were less significantly associated with most of the genes here. *GNB1* and *PRKCZ* are housekeeping genes, and are strongly expressed, while *HMG17* is paralogous to *HMG17L3* in the extended MHC class I region. Of the genes that PML bodies are less associated with than the TAP/LMP locus, the TNF receptor superfamily genes have more limited expression patterns, and are silent in MRC5 fibroblasts (see Figure 3.6). However, the *EIF3S2* and *CASP9* genes are strongly expressed genes, yet do not show the same significant association as *GNB1*, *PRKCZ* or *HMG17*. PML bodies showed less significant association with the *PSMB2* gene, encoding another proteasomal subunit, than the TAP/LMP locus.

Three other non-paralogous genes were studied on chromosome 1. PML bodies were less significantly associated with *CRYZ* and *HF1* genes in gene-poor regions than the TAP/LMP locus, while they were as associated with the *ITPKB* gene (also in a relatively gene-poor region).

To summarise the results of this section, genes that are paralogous to MHC genes are not uniformly associated with PML bodies. Furthermore, the degree of association is not dependent on (a) which chromosome they are on, (b) whether they are within the larger regions of paralogy, or (c) whether they are paralogous to MHC class I, II or III genes.

3.2.3.2 Association of PML bodies with genes encoding proteasome subunits

Several genes encoding proteasome subunits have been described in the section above on paralogous regions. The *PSMB2* and *PSMB4* genes described above were found to be statistically less associated using Student's t-test than the TAP/LMP locus (containing *PSMB8/LMP2* and *PSMB9/LMP7*). In addition to these, the *PSMB5* (chromosome 14), *PSMB10* (chromosome 16), *PSME1/2* (chromosome 14) and *PSMD10* (chromosome X) genes were examined, and found to have PML bodies associate with them to the same degree as TAP/LMP. It must be noted however, that the expression of *PSMB2* and *PSMB4* (as well as *PSMD4*, *PSMD5* and *PSMD10*) is not affected by IFNγ, which may explain the difference in the association of PML bodies with them. It was also noted, however, that most of the proteasome subunit genes reside in relatively gene-rich regions, except *PSMB2*. One possible hypothesis to explain these findings is that PML bodies serve to co-ordinate the transcription of functionally similar genes, by aggregating them to the same nuclear location. Examination of each proteasome-

encoding gene in turn, when detected with the TAP/LMP, showed that they do not preferentially associate with the same PML body (data not shown, see Figure 4.9.B for a similar example in an interferon treated cell).

The data from the above two sections suggest that PML bodies do not associate uniformly with particular sets of functionally related genes, nor are they limited in their association to only a single subset of genes. Rather, the data from loci studied thus far suggest that the association between PML bodies and a locus is related to gene density and transcription.

3.2.3.3 Correlating locus-PML association with transcriptional activity

To test formally whether gene density and/or transcriptional status correlate with the locus-PML associations, the 54 loci described in Section 3.2.1 were reviewed. For each locus, the mmd-locus measurement was compared with the local transcriptional activity, their individual transcriptional status, and the local gene density. These three variables were calculated as follows:

1. **Local transcriptional activity** was calculated by taking the expression data from the Human Transcriptome Map, accurate as on 1 May 2003 (http://bioinfo.amc.uva.nl/HTM-bin/index.cgi/)(Caron *et al.*, 2001). This map uses the SAGE technique on different tissues for analysing genome-wide transcription, as described in Section 1.1.2.3. Expression levels are measured in transcription units, normalised per 100,000 tags in the library. I summed the expression levels of genes within 1 centirays (cR, approximately 500 kb) on either side of the gene tested or the nearest available neighbouring gene, using the GB4 Radiation Hybrid map. As the MRC5 cell line is a fibroblast cell line derived from the lung, relative transcription units were derived for each gene from the averages of data from the normal lung and the skin fibroblast libraries.
2. The **transcriptional level of an individual gene** was determined by semi-quantitative RT-PCR of total RNA isolated from MRC5 fibroblasts. PCR products were quantified by intensities of bands on agarose gels, using commercially available gel image capture software (Labworks, UVP). Transcription levels were expressed as a ratio of the β-actin mRNA levels.
3. **Local gene density** was calculated for 500 kb on either side of the gene tested. For this, the number of genes at each region was obtained from mapping data available at the National Cell Biology Institute (NCBI) gene mapping database

[http://www.ncbi.nlm.nih.gov/] and the Sanger Institute [www.ensembl.org], accurate as on 1 May 2003. Novel and predicted genes, and expressed sequence tags (ESTs) were included. The average of the data from the two sources was calculated.

The mmd-locus scores were analysed against all three variables for each locus using linear regression analysis, which attempts to fit the different variables into a line with an equation:

y=ax+b, where **x** and **y** are the variables, **a** is the gradient and **b** the y-intercept.

Using this analysis, values for R^2 (square of the correlation coefficient) and F (ANOVA score) were obtained. Higher R^2 and F indicated a stronger correlation, with p score values of less than 0.05 being considered a significant correlation. Where appropriate, weighted least squares were used with weights proportional to the inverse of the variance of the mmd-locus score.

First, I analyzed the correlation between the mmd-locus and the **local transcriptional activity** of genes within 2 cR (approximately 1 Mb) surrounding the locus. This genomic distance was chosen, since it was found that genes (*PRKCZ* and *TNFRSF14*) separated by as little as 500 kb can have statistically different associations with PML bodies. The findings show a significant correlation between high local transcriptional activity and association with PML bodies (R^2=0.16, F=9.69, p=0.003) (Figure 3.7.A). This significance persisted after weighting according to the variances. Furthermore, the strength of the association was increased by removing from the data set the centromeric and outlying loci with more than 500 relative transcription units (TAP/LMP, *C4B*, *PSMB10*, *GNB1* and *PRKCZ*), resulting in R^2=0.38, F=25.6, p<0.00001 (Figure 3.7.B).

As the transcriptional activity of a region is dependent on both the local gene density and the transcriptional status of each gene, I wished to determine the relative contributions of each of these factors to the association. Regression analysis showed that there was no significant correlation between the **transcription level of an individual gene** and the association of PML bodies with it (R^2=0.069, F=3.86, p=0.055) (Figure 3.7.C). **Local gene density** was calculated for the 1 Mb surrounding the gene tested. Regression analysis showed a significant association of gene-rich regions with PML bodies compared with gene-poor regions (R^2=0.096, F=5.52, p=0.02) (Figure 3.7.D).

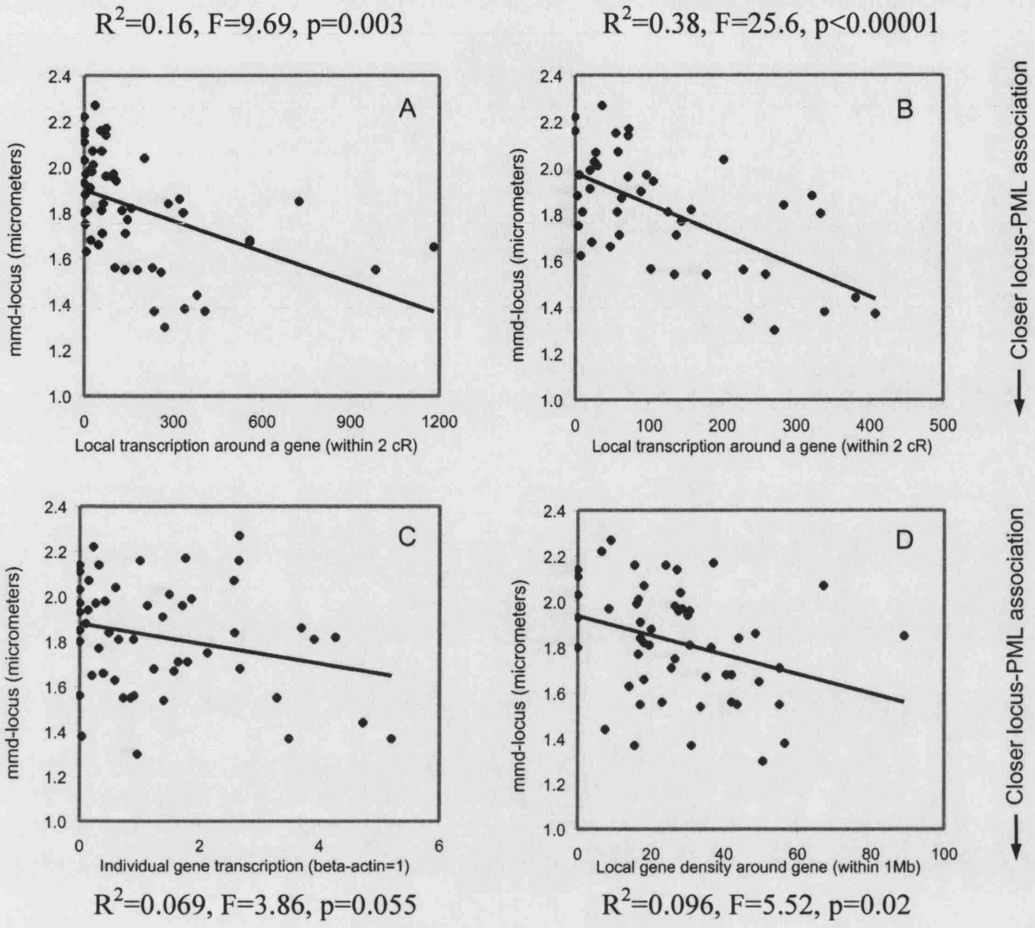

Figure 3.7. Linear regression graphs comparing locus-PML association with different genetic parameters.

Linear regression analysis was performed on the locus-PML distances for different genes, with the local transcriptional activity (A,B), with individual gene transcription (C), and with the local gene density (D). For (B), centromeric and outlying loci were excluded. The locus-PML local gene density and individual gene transcription are calculated as in Figure 2. Each bullet represents a locus. Each graph displays the best line fit for the regression. Association is represented by mmd-locus in micrometres. The local transcription activity, local gene density and individual gene transcription are calculated as in the text. Each bullet represents a locus. Each graph displays the best line fit for the regression.

It was therefore determined that the most important variable is the expression level surrounding the locus, followed by the gene density. My findings also showed that PML bodies associate with genomic regions that have high overall levels of transcription, rather than with individual genes with high transcription levels. For example, genes such as *NOTCH1* in regions of high transcriptional activity, although not being highly expressed themselves, can have strong associations with PML bodies statistically. As shown in Section 3.2.1.3 (where neither *TAP/LMP* nor *C4B* is always closer to a PML body), PML bodies may associate with different genes at different times within a region of high transcriptional activity. This again suggests that the association of a PML body (or PML bodies) with a region is not influenced by one particular gene.

3.2.3.4 Association of PML bodies with genes on the active and inactive X chromosomes

To test further the association of PML bodies with transcriptionally active genomic regions, the genes along the X chromosomes were studied. I first compared the PML bodies around the active and inactive X chromosomes in a female diploid fibroblast cell line (WI-38). The active and inactive X chromosomes territories have been shown to have similar volumes (Eils *et al.*, 1996). The X chromosomes were labelled with whole chromosome paints. The active and inactive X chromosomes were distinguished by the Barr body superimposed on the territory paint, as detected by 4'6'-diamino-2-phenylindole (DAPI) or propidium iodide (PI). The spatial relationships of whole chromosome territories with PML bodies were determined visually using 3D stacks of each cell. The number of PML bodies in direct contact with each chromosome territory was counted in each cell by two independent observers, and the means of the scores taken for subsequent analysis. Comparisons between the number of PML bodies in contact with each territory were analysed using the paired Students' t-test for equal variances. I found more PML bodies around the active X chromosome, which was statistically significant (mean number of PML bodies around Xa = 1.34, Xi = 0.78, $p = 0.0001$, number of cells, $n = 51$).

As the inactive X chromosome (Barr body) is often noted to be located in the nuclear periphery (Dyer *et al.*, 1989), it may be argued that the association of the active chromosome X with PML bodies is due to its more interior position. Therefore, I also examined the p and q arms of the two different X chromosomes separately. It was found statistically that for the q arm there were more PML bodies associated with the active X

chromosome, but the same was not observed for the p arm. (Mean number of PML bodies around Xpa = 0.75, Xpi = 0.63, p = 0.17; Xqa = 0.88, Xqi = 0.48, p=0.004; n=48). This result could be explained by the fact that the pseudoautosomal regions (containing genes which are expressed on both X chromosomes) lie predominantly in the p arm, while the expression of the genes in the q arm of the X chromosomes is mostly from the active X chromosome. I also did not observe a more interior nuclear position of the Xp arm compared with the Xq arm.

Specific loci along the active and inactive X chromosomes were then studied. ImmunoFISH experiments were performed to detect PML bodies and different loci along chromosome X. Simultaneous staining with DAPI or propidium iodide enabled alleles belonging to the inactive X chromosome to be distinguished (Figure 3.8.A). The paired Student's t-test was used as previously shown, except that, since the md for the active/inactive X alleles were being compared, each allele was considered separately. PML bodies were found to be significantly more closely associated with the homologues of six of eleven genes studied (*G6PD, GLA, LAMP2, UBE2A, NAP1L3, and PSMD10*) on the active X-chromosome than those on the inactive X-chromosome, using paired t-tests. In contrast, no significant difference was found between the active and inactive copies of the *ALTE, DMD* and *USP9X* genes (all located in or around the pseudoautosomal regions on Xp) (Carrel *et al.*, 1999), in their association with PML bodies. Similarly, no difference was found between the two copies of the chromosome X centromeres. Finally, PML bodies associated with the *XIST* genes on each X-chromosome to a similar extent. The *XIST* gene, although only expressed from the inactive X chromosome (Brown *et al.*, 1991), lies in a region not known to be a pseudoautosomal region on the Xq arm. (See Figure 3.8.B and Appendix C.3 for the results.)

Obviously, the two X chromosomes have the same gene densities and gene sequences. The differences between them lie in the transcription of the genes and the epigenetic and chromatin features, such as histone acetylation and methylation levels. These factors thus appear to be more important in the association with PML bodies rather than gene density alone.

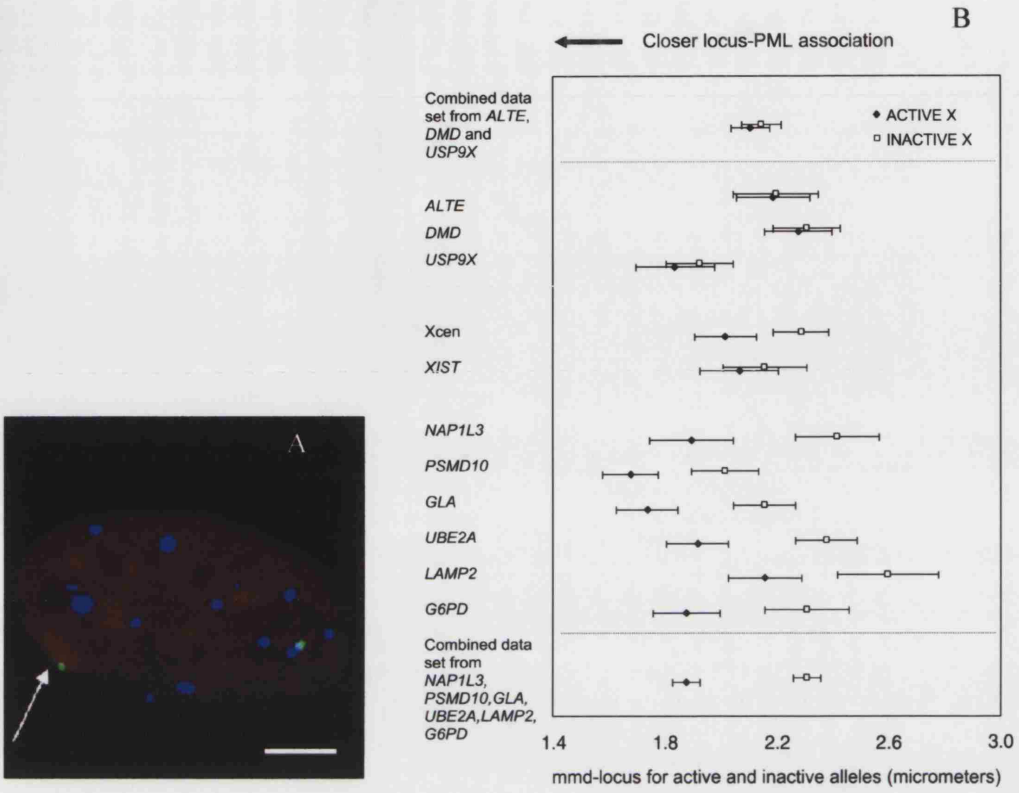

Figure 3.8. Association of PML bodies with loci on the active and inactive X chromosomes.

A: WI-38 fibroblast cell nucleus showing the *PSMD10* gene (green) on the X-chromosome, PML bodies (blue), and propidium iodide counterstain (red). The inactive copy of the gene is distinguished by the Barr body (arrowed). The solid line represents 5 micrometres.

B: Comparison was performed for the locus-PML distances for loci along active and inactive X chromosomes. The locus-PML association is represented as the mmd-locus distances for the active and inactive copy of each locus, with error bars showing 1 standard error. Results for loci in and out of the pseudoautosomal regions are pooled and displayed at the top and bottom of the figure respectively.

3.2.3.5 Locus-PML association during the cell cycle

Shiels *et al* showed that association of PML bodies with the TAP/LMP locus remains the same in the G0/G1 and S-phases of the cell cycle (Shiels *et al.*, 2001). I have now extended these studies to other regions of chromosome 6. These include 6p22 which contains genes encoding histone proteins, of which a large proportion are transcribed at high levels predominantly in S-phase (van der Meijden *et al.*, 2002), as well as the chromosome 6 centromere and the 6p24 region.

Cells in G0/G1 and S-phases were distinguished by incorporation of BrdU (Figure 3.9.A and B). Cells in G2 detected by a double signal per allele were excluded. I found that cells in S-phase had more PML bodies, and hence a decrease in the PML-PML distances. Adjusting for this, and using unpaired t-tests for each of the loci, no significant difference was found between these phases of the cell cycle for associations of PML bodies with the TAP/LMP, chromosome 6 centromere and 6p24 loci. In contrast, the 6p22 region showed a significant increase in association in S-phase compared to G0/G1-phase (Figure 3.9.C and Appendix C.4).

Thus, for the majority of loci studied, there appeared to be no statistical increase in association between G0/G1-phases and S-phases of the cell cycle. The notable exception is the histone gene cluster on 6p22, which, as mentioned, is expressed predominantly in S-phase. This further links the association of PML bodies with transcriptional activity of a clustered region. Since the number of PML bodies increases in S-phase, the role of PML bodies in DNA replication will be explored further (Chapter 5).

3.2.4 Other considerations in analysing locus-PML association

3.2.4.1 Locus-PML association and gene position within chromosome territories

This laboratory has previously been shown that in a proportion of MRC5 cells, the MHC region on chromosome 6 is positioned outside the chromosome territory as delineated by whole chromosome paint probe (Volpi *et al.*, 2000). Furthermore, the proportion of cells with this external positioning of the MHC is increased in cell types where the MHC genes are active, such as B-lymphoblastoid cells and MRC5 fibroblast cells treated with IFNγ. This configuration was not observed in the centromere or 6p24 region. This prompted me, as well as others, to study whether there is a link between the

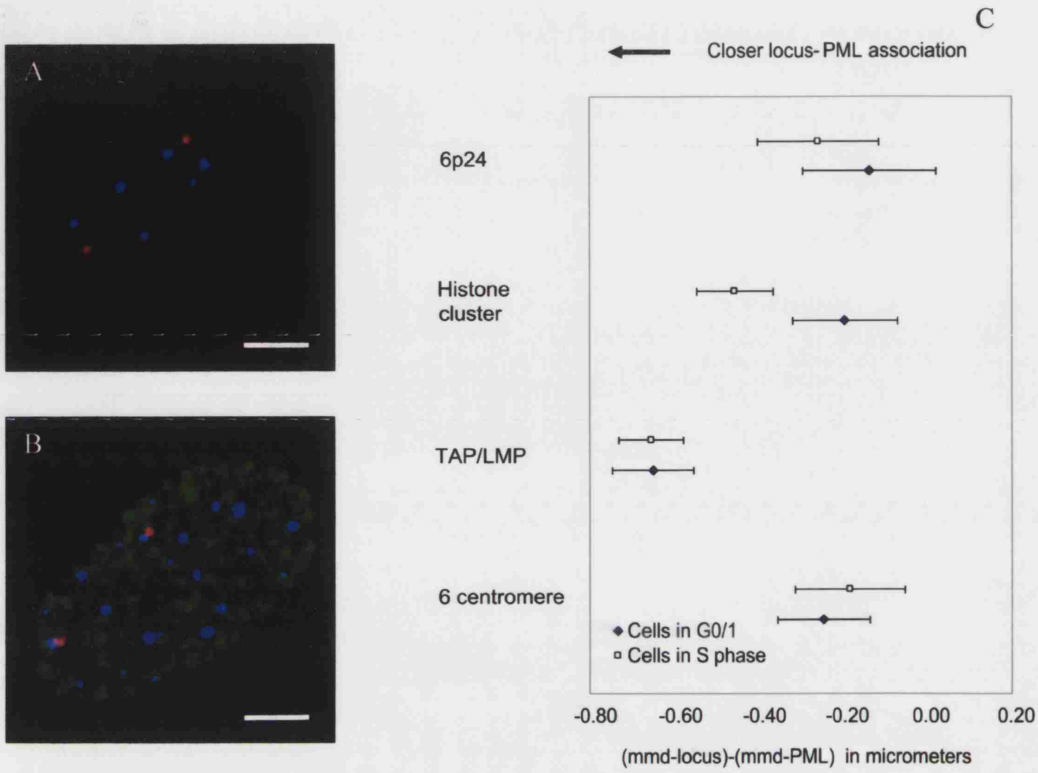

Figure 3.9. Association of PML bodies with loci along chromosome 6, in cells in S phase compared with cells in G0/1 phase.
A and B: MRC5 cell nuclei showing the histone gene cluster (red) at 6p22, PML bodies (blue), and incorporated BrdU (green) [A: cell in non-S (G0/G1) phase, B: cell in S phase]. The solid line represents 5 micrometres.
C: Locus-PML distances for different loci were compared between cells in S phase versus G0/G1 phase. The (mmd-locus) – (mmd-PML) on X-axis represents the distance to which a locus is further or closer to the nearest PML body compared with the PML-PML distance, with error bars showing 1.4 standard errors. T-tests were performed between the cells of G0/G1 and S phases for the 4 loci. Only the histone cluster on 6p22 shows a significant difference in gene-PML association between cells in S versus G0/G1 phase.

position of the gene relative to the chromosome territory and its association with the PML bodies. Williams *et al* showed that in differentiating keratinocytes, there is an increase in both the external signals of the transcriptionally active EDC region and the association with PML bodies, compared with lymphoblasts (in this paper, direct contact with PML bodies by visual counting was used) (Williams *et al.*, 2002). However, when the two variables were analysed in relation to each other in this study, it was reported that external locus signals were not more likely to be associated with PML bodies.

I therefore wished to confirm these findings using selected loci that show different PML body-associations as assessed by mmd measurements. ImmunoFISH experiments were performed to detect PML bodies, the loci under investigation, and their corresponding chromosome territories by whole chromosome painting. Each cell was examined by two independent observers as a 3D stack of images, and the position of a locus was scored to be either peripheral to its parent territory (if at least part of it is touching the edge of the territory), internal, or external to it (Volpi *et al.*, 2000). The locus was also visually scored for direct contact with a PML body (see Figure 3.10.A and B for examples). For each locus, a 3 by 2 table of contingencies was formed, which allows the relationship between its position and its contact with PML bodies to be statistically analysed using the Chi-squared test. To test the hypothesis that the more external a locus was, the more likely it would have a PML body associated with it, the Chi-squared test for trend was also applied. Here, the position of the loci with respect to their territories was scored from 1 (internal) to 3 (external). The test for trend determines whether an increased score correlated to more frequent PML contact. For a subset of loci, measurements of the md-locus were also obtained (each allele in a cell was considered as a separate entity). Comparisons of md in loci that were external, peripheral or internal to their territories were also compared using regression analysis of the mmd versus territory localisation (again scored from 1 to 3).

The TAP/LMP locus, the 6p24 region, and the centromere of chromosome 6 were each visualised together with the chromosome 6 territory and PML bodies. I found that the position of these regions with regard to the chromosome territory did not correlate with whether they were in direct contact with PML bodies (Table 3.4). It was noted that PML bodies associated with loci, even in the occasional cell when they were in the interior of their territories. I then examined the (more strongly PML-associated by mmd) genes *HMG17* and *GNB1* (with the corresponding chromosome 1 paint), and *PSMB7* and

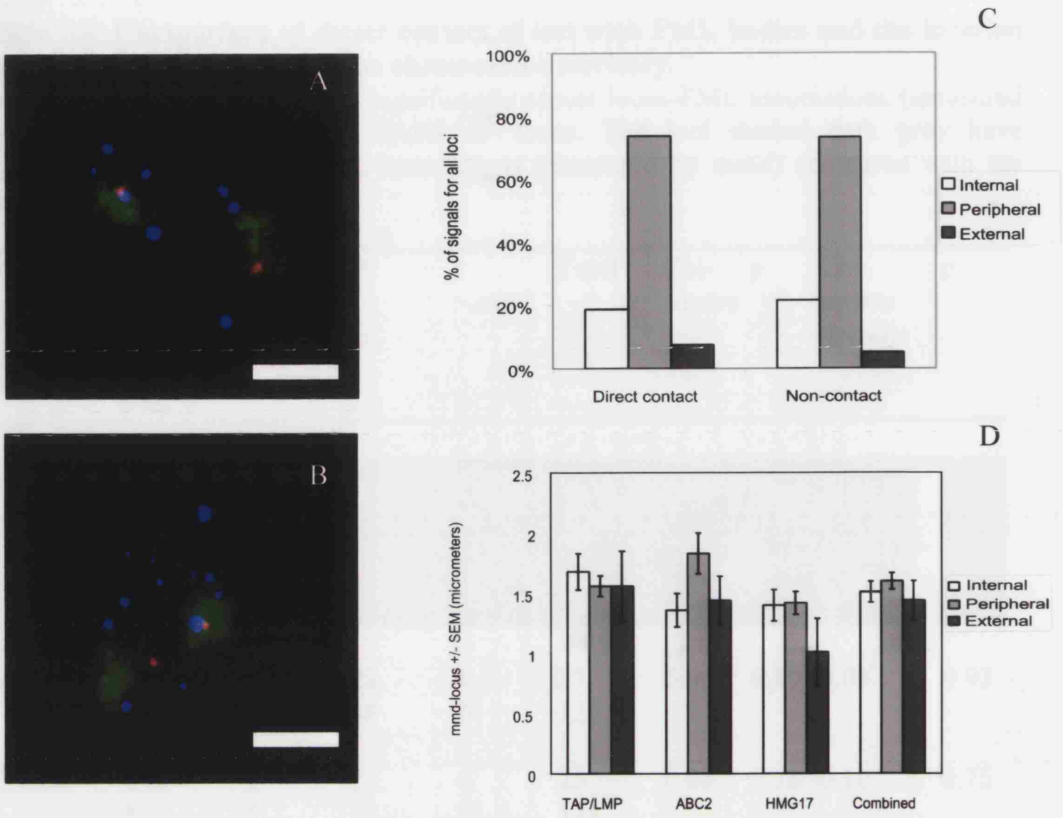

Figure 3.10. Comparison of locus-PML associations relative to the positions of the loci within their chromosome territories.

A: MRC5 cell nucleus showing the TAP/LMP locus (red), chromosome 6 territory (green), and PML bodies (blue). Both TAP/LMP signals are peripheral to the territory, with one locus in contact with a PML body and the other not.

B: MRC5 cell nucleus, showing one TAP/LMP is external to the territory and is not in contact with a PML body, the other allele is internal to its territory, but is in contact with a PML body. The solid line represents 5 micrometres.

C: Comparison of locus-PML body associations (pooled from 10 different loci) relative to locus positions within their chromosome territories. The TAP/LMP locus, the 6p24 region and chromosome 6 centromere were analysed, as well as seven other loci (*HMG17, GNB1, TNFRSF14, EIF3S2, TRKB, ABC2, and PSMB7*) on chromosomes 1 and 9. Their association with PML bodies was not dependent on their position relative to the territory.

D: Comparison of the mmd-locus for 3 loci (TAP/LMP, *ABC2, HMG17*) relative to locus positions (external, peripheral or internal to their respective chromosome territories). Error bars show 1 standard error. Comparisons of loci were made using linear regression models. Analysis of these three data sets did not reveal any statistical differences or trends in the locus-PML association for loci in different positions.

Table 3.4. Comparison of direct contact of loci with PML bodies and the location of the locus signal relative to the chromosome territory.
The loci shaded light grey have significantly closer locus-PML associations (measured by mmd) compared with the TAP/LMP locus. The loci shaded dark grey have significantly weaker locus-PML associations (measured by mmd) compared with the TAP/LMP locus.

Loci	Visually in contact with PML?	E	P	I	Total	Chi-square	p	Chi-square (trend)	p
ABC2	Yes	6	27	3	36	0.48	0.49	0.82	0.37
	No	14	75	17	106				
HMG17	Yes	3	28	5	36	2.34	0.13	3.01	0.08
	No	6	67	33	106				
TAP/LMP	Yes	2	21	4	27	0.06	0.80	0.01	0.93
	No	11	88	24	123				
PSMB7	Yes	1	22	4	25	0.08	0.78	0.10	0.75
	No	5	91	25	121				
GNB1	Yes	1	16	10	27	0.45	0.50	0.68	0.41
	No	4	74	27	105				
6cen	Yes	2	13	1	16	2.43	0.12	2.94	0.09
	No	3	61	20	84				
6p24	Yes	0	8	2	10	0.01	0.93	0.02	0.89
	No	1	70	15	86				
TRKB	Yes	0	8	6	14	NA	NA	NA	NA
	No	0	77	7	84				
TNFRSF14	Yes	2	7	4	13	0.99	0.32	0.04	0.84
	No	7	82	26	115				
EIF3S2	Yes	1	7	2	9	2.22	0.14	0.71	0.40
	No	1	63	28	93				

E – external loci, P – peripheral loci, I – internal loci.

ABC2 (both with chromosome 9 paint), and found that although all 4 genes were frequently in contact with PML bodies, *GNB1*, *HMG17* and *PSMB7* had fewer external signals than *ABC2* . This suggests that the two phenomena, external signals and contact with PML bodies, can exist separately from each other. In contrast, the (less PML associated by mmd) *TRKB* (chromosome 9), *TNFRSF14* and *EIF3S2* (both chromosome 1) all have relatively few external signals.

Chi-square tests for loci tested here (except *TRKB*, which had insufficient external signals for the Chi-square test to be applied), were not significant. This suggested that no correlation exists between signal localisation in relation to the chromosome territory and their contact with PML bodies (Table 3.4, pooled data shown in Figure 3.10.C). Likewise regression analysis for mmd of three of the loci, TAP/LMP, *ABC2* and *HMG17*, as well as the pooled data did not show any significant correlation between external, peripheral or internal signals, and the mmd distances measured (Figure 3.10.D, Appendix C.5). These data imply that signals that are internal or peripheral to the parent chromosome territory were just as likely to be associated with PML bodies as external signals. My results therefore agreed with, and extended, the study by Williams *et al* (Williams *et al.*, 2002).

3.2.4.2 Association of PML bodies with whole chromosome territories

One possible interpretation of the results that I have obtained is that PML bodies associate to varying degrees with specific whole chromosomes due to their different radial positions in the nucleus, rather than with individual genes. This may in turn be related to either overall gene density or the physical size of each chromosome territory (see Section 1.2.1.1). This was a consideration in the study using the active/inactive X chromosomes.

To analyse this further, the number of PML bodies in contact with chromosomes 18 and 19 were visually counted. These chromosomes have different gene densities but similar genetic sizes (chromosome 18 is approximately 76 Mb long, chromosome 19 is 64 Mb). Furthermore, the chromosome 19 territory is recognised to have a more interior nuclear position than chromosome 18 (Croft *et al.*, 1999). The number of PML bodies in direct contact with different chromosome territories was counted as for the study on the X chromosome. Analysis of the findings showed that more PML bodies were in contact with the relatively gene-rich chromosome 19 compared with chromosome 18, (mean

number of PML bodies around chromosome 18=1.93, 19=2.18, $p<0.05$, n=60 and 63 respectively). Analysis using the unpaired t-test showed that this result was significant.

To determine whether this difference is due to nuclear positioning or gene density, individual genes were then studied from both chromosomes, and compared with the TAP/LMP locus using the mmd method. PML bodies were found statistically to be as associated with two genes on chromosome 19, *C3* and *KIR3L2* (both in gene-rich regions) as the TAP/LMP locus. In contrast, PML bodies were less significantly associated with the *TGIF* gene on 18p than TAP/LMP, while they were as associated with *LMAN1* on 18q. Both regions on chromosome 18 are moderately gene-dense and have comparable gene activity, thus the different associations might be due to non-transcription related factors or to subtle local transcription differences. Importantly, it was found that PML bodies were less significantly associated with the centromeres on both the chromosomes, than with the TAP/LMP locus.

Thus, the disparate nuclear positions of chromosomes 18 and 19 are insufficient to account for a general increase in association of PML bodies with genes on chromosome 19. The transcriptional activity and gene density of different regions of a chromosome is therefore thought to be the basis of increased association of chromosome 19 with PML bodies.

3.2.4.3 Locus-PML association in relation to other nuclear domains

Shiels *et al* previously showed that the MHC associated independently with both Cajal bodies and with PML bodies (Shiels *et al.*, 2001). As PML bodies and SC-35 domains (which includes both SFC and PF) both lie in the interchromosomal space, and are often found adjacent to each other (see Section 1.2.2.3), one possible explanation for our observation is that PML bodies associate with genes by proxy to the SC-35 domains. The SC-35 domains (specifically labelled with anti-SC-35 antibodies) in the interphase nuclei have also been shown by Lawrence and colleagues to associate to different extents with specific genes that encode proteins having structural or cytoskeletal functions (Smith *et al.*, 1999; Xing *et al.*, 1995). In particular, the *COL1A1*, *LMNA* and *ACTB* genes were reported to be associated with SC-35 domains while *LAMB1* was not. In these studies, assessment of association between SC-35 domains and genes was performed using direct visual contact, as these domains are more diffuse and do not often form discrete bodies.

I therefore wished to determine if some of the genes I studied have different associations with SC-35 domains. I initially used probes directed against TAP/LMP, 6p24, and the chromosome 6 centromere, and antibodies against SC-35 domains, and found that more signals from the TAP/LMP locus were in direct contact with SC-35 domains, as compared with the 6p24 and centromeric regions (see Table 3.5).

As a reverse approach, I wished to determine if the four genes above (*COL1A1*, *LMNA*, *ACTB* and *LAMB1*) studied by Lawrence and colleagues had varying associations with PML bodies. Using direct visual contact assessment, I confirmed the previous results by Lawrence and colleagues (Smith *et al.*, 1999). I then examined the association of these four genes with PML bodies using mmd measurements and t-tests, and found that while PML bodies associate with the *COL1A1*, *LMNA* and *LAMB1* genes to the same extent as the TAP/LMP locus, the *ACTB* gene was less significantly associated. The findings on the *LAMB1* and *ACTB* genes therefore differ from what would be expected if the association of PML bodies with these genes was related to the proximity of SC-35 domains.

Next, chi-square tests were performed to compare directly the association of loci with SC-35 domains and their association with PML bodies (both by direct visual counting). All seven loci mentioned above were re-tested using immunoFISH, with PML bodies and SC-35 domains labelled simultaneously. When the number of signals in direct contact with PML bodies and SC-35 domains were counted, it was found that the loci may be in contact with either PML bodies or SC-35 domains independently. That is, loci that were in contact with SC-35 domains were not necessarily in contact with PML bodies, and *vice versa*. The Chi-square tests on 2x2 contingency tables show that for the majority of these genes (with the exception of the chromosome 6 centromere and the *ACTB* gene), their association with PML bodies is unrelated to their association with SC-35 domains (Table 3.5).

The research described above in Sections 3.2.2.1 – 3.2.2.3 is important in showing that the association of certain loci with PML bodies is unlikely to be a secondary effect of other nuclear associations, for example with SC-35 domains or Cajal bodies. The effects of whole chromosome territories and their radial nuclear positions have also been examined. Other nuclear factors may also influence locus-PML associations, but those tested represent the most likely confounding factors, since various genes have been

Table 3.5: Comparison of direct contact of loci with PML bodies and the contact of loci with SC-35 domains.

The loci shaded grey have significantly further locus-PML associations (measured by mmd) compared with the TAP/LMP locus. The other loci (*COL1A1, LMNA, LAMB1*) were as associated as the TAP/LMP locus.

Loci	Visually in contact with PML?	Contact with SC-35 domains	Not in contact with SC-35	Total	Chi-square	p
TAP/LMP	Yes	11	3	14	1.575	0.455
	No	71	36	107		
COL1A1	Yes	11	0	11	2.031	0.362
	No	51	2	53		
LMNA	Yes	8	3	11	1.379	0.502
	No	35	21	56		
LAMB1	Yes	2	4	6	0.736	0.692
	No	16	45	61		
6cen	Yes	7	4	11	7.009	0.030
	No	14	34	48		
6p24	Yes	0	2	2	1.557	0.459
	No	7	52	59		
ACTB	Yes	9	3	12	9.898	0.007
	No	16	36	52		

found the associate with the above-mentioned bodies or with respect to their chromosome territory or nuclear positions.

3.2.4.4 PML bodies in relation to sites of transcription and RNA transcripts

To determine whether PML bodies are themselves sites of transcription, I used RNA-FISH simultaneously with DNA-FISH and immunofluorescence to visualise RNA transcripts for 3 genes, the genomic loci themselves, and PML bodies, respectively. Loci tested were TAP/LMP, and the *HSPA5* and *COL1A1* genes. These genes were chosen as they showed comparable associations with PML bodies, and are actively transcribed. Since the TAP/LMP locus contains the co-ordinately expressed *PSMB8/LMP2, PSMB9/LMP7, TAP1* and *TAP2* genes, and genomic probes covering the whole region were used for the RNA-FISH, the RNA signals represented all four RNA transcripts. To induce the expression and facilitate RNA-FISH, cells were either treated with IFNγ at 200 U/ml, for detecting the TAP/LMP genes, or subjected to heat shock (44 °C for 1 hour) for the *HSPA5* gene. It must be noted that while the presence of an RNA signal indicates active transcription, the reverse cannot be implied, as RNA is easily degraded.

As expected, the RNA transcripts were found to be adjacent to and extending away from the DNA probe signals. Although a high proportion of the transcripts were adjacent to PML bodies, some were not in direct contact with PML bodies (Figure 3.11, Table 3.6A). The DNA probe signals were also examined with respect to contact with PML bodies in the cells that were shown to have an RNA signal (i.e. they were being expressed at the time of fixation). It was found that for actively transcribed genes, a large proportion of genomic signals were in contract with PML bodies, but this was not invariably so. This was not unexpected, as these three loci were known to associate significantly with PML bodies as measured by the mmd method. Furthermore, for *TAP/LMP* and *HSPA5* genes, the interferon and heat-shock treatment of the cells resulted in an increase in the number of PML bodies. Interestingly, in genes that had RNA signals, more RNA signals were in contact with PML bodies compared with the corresponding genomic loci. However, this is thought to be due to the fact that the RNA signals tended to be bigger and more diffuse than the DNA signals.

These findings suggest that neither PML bodies nor their immediate surroundings serve specifically as transcription foci, at least for these three loci. I then decided to see if

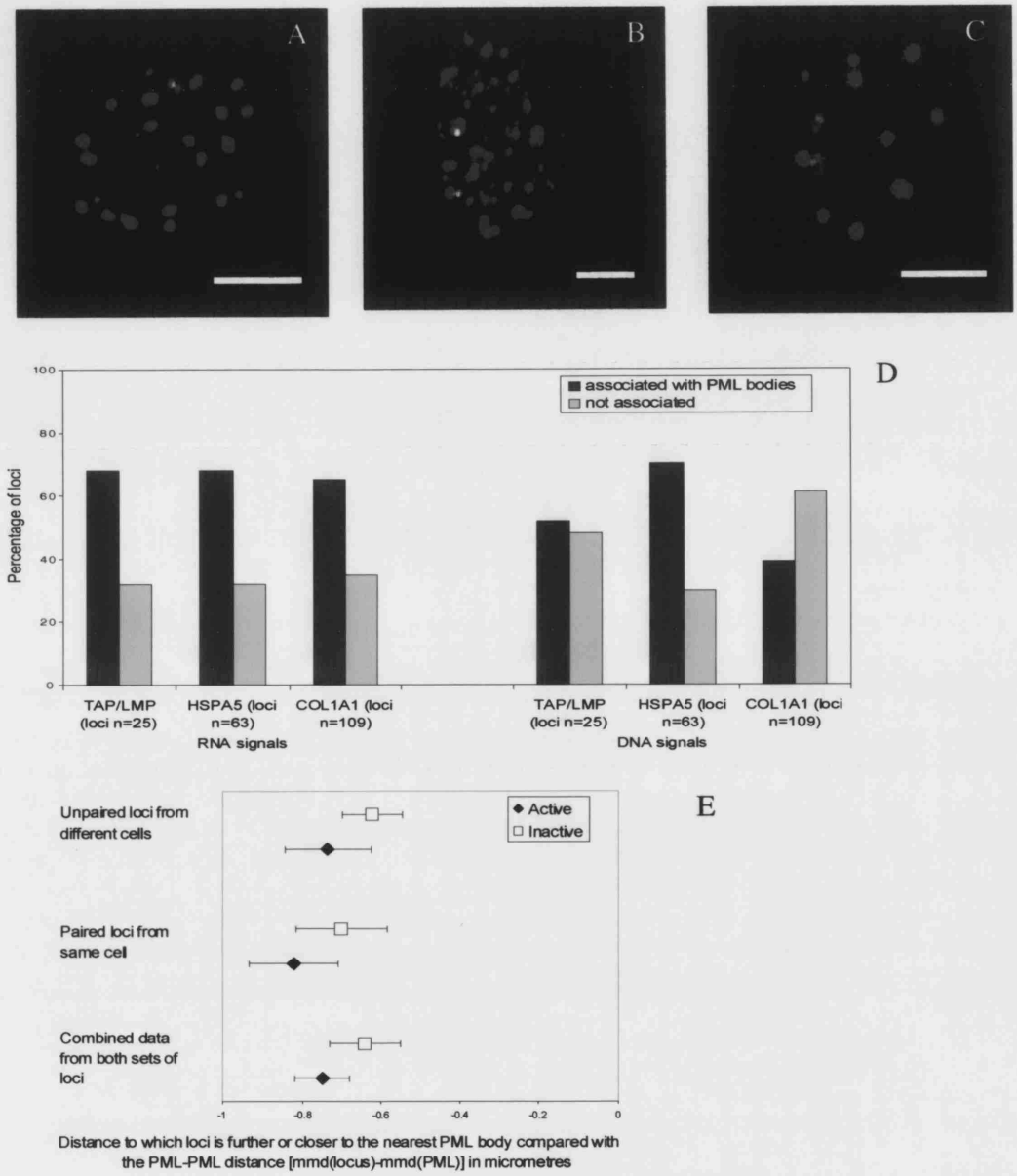

Figure 3.11. Association of RNA transcripts and their sites of transcription with PML bodies.

MRC5 cells showing the RNA transcript signals (green), corresponding genomic loci (red) and PML bodies (blue) for the TAP/LMP (A), *HSPA5* (B) and *COL1A1* (C) loci. The TAP/LMP locus comprises the genes *PSMB8/LMP2, PSMB9/LMP7, TAP1* and *TAP2*. The solid line represents 5 micrometres.

D: The proportion of RNA and genomic signals (in cells with detectable RNA signals) which were in direct contact with a PML body for the three loci, compared with those not in direct contact. The DNA signals in loci containing RNA signals reflect the sites of transcription.

E: The locus-PML association of DNA signals for loci with and without RNA signals. Transcriptionally active loci tended to be closer to PML bodies, but this did not reach statistical significance. The error bars for the unpaired loci and combined data show1.4 standard errors, the error bars for the paired loci show 1 standard error.

Table 3.6.A: Results of RNA-FISH data for the *COL1A1*, *HSPA5* and TAP/LMP loci.

Locus	Total number of cells	Number of loci (gene)	RNA signal positive	RNA loci in direct contact with PML body	DNA loci in direct contact (RNA positive)	DNA loci in direct contact (all loci)
COL1A1	93	186	109	71(65%)	42(39%)	60(32%)
HSPA5	73	146	63	43 (68%)	44(70%)	97(66%)
TAP/LMP	31	62	25	17 (68%)	13(52%)	25(40%)

Table 3.6.B: Comparison of mean minimum gene-PML distances between the *COL1A1* alleles showed an RNA signal and those which did not.

RNA signal present	Number of loci	mmd for loci	mmd PML-PML	t statistics comparing alleles with and without RNA signals
Yes	109	1.32μm	2.09μm	)
				) t= -1.35, p=0.18
No	77	1.57μm	2.21μm	)

Table 3.6.C: Comparison of mean minimum gene-PML distances between the *COL1A1* alleles in cells which had only one allele showing an RNA signal.

Number of cells	mmd for loci with RNA signals	mmd for loci without RNA signals	mmd PML-PML	t statistics comparing alleles with and without RNA signals
19	1.25μm	1.37μm	2.31μm	t= -0.82, p=0.42

Table 3.6.D: Comparison of mean minimum gene-PML distances between different populations of cells in which either both *COL1A1* alleles showed RNA signals or both alleles did not.

RNA signal present	Number of cells	mmd (gene)	mmd (PML)	mmd(gene) – mmd(PML)	t statistics comparing alleles with and without RNA signals
Yes	45	1.33μm	2.07μm	-0.73μm	)
					) t= -1.13, p= 0.26
No	29	1.64μm	2.26μm	-0.62μm	)

actively transcribed genes were also closer to PML bodies than those not being transcribed. Since heat shock causes PML bodies to disperse into a microspeckled and diffuse pattern, statistical analysis based on distance measurements was not possible for the *HSPA5* gene. The TAP/LMP locus was less actively transcribed, even in interferon-induced cells and was also not suitable for this analysis.

Using the *COL1A1* gene however, I was able to determine for each allele in the set of cells, its minimum distance to the nearest PML body (md_{col1a1}). *COL1A1* alleles were then grouped into whether a RNA transcript was detectable or not. (Although, as mentioned above, the lack of visible RNA transcripts does not prove that transcription is not occurring.) Comparison of the mmd distances for the genes which had RNA signals with those without signals showed no statistical difference in their association with PML bodies (Table 3.6.B).

To confirm these findings, three different populations of cells were derived, depending on whether both alleles expressed detectable RNA, only one expressed, or both alleles did not express. A paired t-test was carried out in the population of cells where only one allele was expressed; this showed no statistical difference in the gene-PML distances (Table 3.6.C). The unpaired t-test was carried out for the other two sets of cells, taking into account the differences in PML-PML distances. No significant difference was again seen in the PML body association between expressed and unexpressed cells using the unpaired t- test (Table 3.6.D). Finally, a combination of both t-statistics was used after weighting for their variances with similar results ($t=1.62$, $p=0.10$). These results again suggest that closer gene-PML associations are not due to the active expression level of a single gene at the time of fixation.

3.3 Discussion

Previous work done in the laboratory showed that PML bodies associated preferentially with the MHC (Shiels *et al.*, 2001). Here, I have examined whether PML bodies are associated with other genomic regions, and have attempted to determine the biological basis for such associations.

In the experiments, PML bodies were found to be more associated with certain loci than others when measured by the mmd method, but this association was retained even when those loci that touch PML bodies are excluded from the calculations. This result might

be explained by the fact that chromatin can move up to 2μm (Chubb *et al.*, 2002), and genes which are statistically closer might therefore associate intermittently with PML bodies more frequently, or be in the process of being recruited to/from PML bodies. This association may be related to transcription. However, RNA-FISH experiments showed that when certain genes which show a high degree of association with PML bodies are being transcribed, they and their mRNA transcripts are not always adjacent or in direct contact with PML bodies. This is similar to a previous report in which Cajal bodies associate with histone genes, but are not necessarily sites for transcription (Shopland *et al.*, 2001). It is therefore proposed that a locus may form a statistically significant, but ***indirect*** association with PML bodies as a result of its location within a nuclear compartment in which a PML body is likewise positioned. Taken together, the data presented in this chapter show that PML bodies are located within or in close proximity to transcriptionally permissive nuclear compartments (Isogai and Tjian, 2003), but they do not necessarily colocalise with transcription sites.

By examining multiple genomic regions with different features (54 loci on 9 autosomal chromosomes, covering over 20 distinct genomic regions), it was found that PML bodies associate with genomic regions of high transcriptional activity. This in turn is a function of both the gene density and the proportion of genes that are active. PML bodies do not show significant associations with single genes that are highly expressed alone.

The hypothesis was therefore that PML bodies associate specifically with regions of high transcription. This was then tested formally using linear regression statistics. The regression model showed some loci to be outside the line fit, suggesting that other factors may play a role in their association with PML bodies:

1. PML bodies may associate with certain regions as a result of non-transcription-related activities. For example, previous studies have shown that PML bodies can associate with centromeres under specific conditions, such as in a subset of cells in S/G2-phase and when cells are treated with MG-132, a proteasome inhibitor (Everett *et al.*, 1999a). This study did not differentiate specific chromosomes, but it may be that different centromeres associate to different degrees with PML bodies. In my study, I found PML bodies were as significantly associated with the chromosome 9 centromere as with the TAP/LMP locus in the MHC. This was in contrast to the other centromeres tested. Chromosome 9 centromere was found to have unique

properties. Specifically, Heat Shock Factor-1 (HSF1) bodies were found to interact specifically with the centromere of chromosome 9 following heat shock (Jolly *et al.*, 2002). HSF1 protein is also noted in a separate study to be redistributed to PML bodies with stress (Hong *et al.*, 2001), and it remains to be seen if the two observations together explain my results for this centromere. (It must be noted however, that the centromere on chromosome 9 is adjacent to a highly gene-rich region, and the closeness of the centromere to PML bodies may be due to the nearby region. The CREB3 and PAX5 loci for example, are located at a gene-rich band (9p11) near the centromere, and are both found by the mmd method to associate with PML bodies.)

2. Secondly, the nucleus has a limited volume, and associations can exist due to the tightly packaged space. Genes may be found to associate with a PML body due to the influence of neighbouring genes on the same chromosome, or even on neighbouring chromosomes. It remains to be seen whether transcriptionally active genomic regions from adjacent chromosome territories co-localise in the above-mentioned functional compartments, given that there is no consensus about the determinants influencing chromosome ordering in interphase (discussed in Chapter 1).
3. The input used for the regression studies is limited by the accuracy of the mapping data. The mapping and transcriptome data currently available bioinformatically are still incomplete, and gene positions may be incorrect. In addition, the 1 Mb limit for measuring gene density and local transcriptional activity remains arbitrary, and different chromosome packaging may exist in different regions, rendering linear genetic distances less meaningful.
4. Finally, using this regression method, I am unable to exclude the possibility that PML bodies interact with only a subset of genes. This will be discussed later, and in Chapter 4.

The organisation of PML bodies in relation to transcriptionally active regions is further supported by the association of PML bodies with many genes on the active X-chromosome compared with their homologues on the inactive X-chromosome, and with replication-dependent histone genes on chromosome 6 in S-phase cells compared with those in G0/G1-phase cells. Although both the previous study using interferon treatment and the RNA-FISH results suggest that the association of PML bodies with a locus does not form in response to rapid changes to transcription, I nevertheless found an increased association of PML bodies with the histone cluster in S-phase. While this may be

contradictory, it must be noted that cells in S-phase show a distinct change in pattern and numbers of PML bodies, suggesting a particular plasticity of PML bodies during this particular period. This will be explored further in Chapter 5. Alternatively, changes in transcription in a cluster of genes may influence PML body association to a greater extent than that of individual genes.

Another aim of my experiments was to exclude any statistical confounding factors, that is, that the association of PML bodies with genomic regions is due to a third factor. I showed that the position of a locus towards a more peripheral or external conformation with respect to its chromosome territory (as compared with an interior position) did not correlate with increased PML association, despite PML bodies being found mostly in the interchromosomal space (Bridger *et al.*, 1998). The possibility that the association of loci with PML bodies was due to their proximity to certain other nuclear inclusions, in particular Cajal bodies as previously described (Shiels *et al.*, 2001) or SC-35 domains, has also been excluded. It may be argued that PML bodies associate with specific loci due to their radial position in the interior of cell nucleus. Our data on the pseudoautosomal region of the inactive X chromosome, and on individual loci along chromosomes 18 and 19, suggest otherwise. Thus, the locus-PML association appears to be a *bone fide* finding.

Two interrelated questions arise from the findings so far. Firstly, what are the dynamics or mechanics forming such associations; and secondly, what are the underlying factors that influence the associations?

3.3.1 Possible mechanism for genome-PML association

The mechanism that allows PML bodies to associate with specific loci is beyond the aims of this thesis, and remains an area of speculation. Such associations may be due to movements (or at least changes in position) of either the PML bodies or the different loci.

As mentioned previously, **PML bodies** are bound to the nuclear matrix and are generally immobile (Stuurman *et al.*, 1992), although a subset of nuclear bodies are able to move in an energy dependent manner (Muratani *et al.*, 2002). More recently, PML bodies have been shown by live cell imaging to form by budding from a parent PML body (Eskiw *et al.*, 2003). In another study from the same group, it was shown that

under certain circumstances (such as transcriptional inhibition), PML bodies detach from anchored points (proposed to be chromatin), and become more mobile (Eskiw *et al.*, 2004). While it is possible that such movements result in associations with certain specific genomic regions, the static nature of the majority of PML bodies would suggest this is not the basis of a large-scale PML body organisation resulting in multiple gene-PML associations.

Another neglected or even discounted (Eskiw *et al.*, 2003) possibility is that new PML bodies may be formed *de novo* by the accumulation of constituent proteins in the interchromosomal domain (either from newly synthesised protein, or from the pool in the nucleoplasm). One scenario is that the concentration of PML protein in a specific location within the nucleus reaches a critical concentration, triggering nucleation of a PML body (Hancock, 2004). This is seen in the formation of PML bodies in G1-phase of the cell cycle (Koken *et al.*, 1995). Could it be that the organisation of PML bodies with respect to transcriptionally active regions is established as a cell exits mitosis and general transcription initiates? If so, can subsequent transcriptional events also cause new PML bodies to form at nearby sites during the remainder of interphase? Apart from early G1, *de novo* formation of PML bodies is also seen in acute promyelocytic leukaemia cells with the PML-RARα translocation, when treated with ATRA (Zhu *et al.*, 1995). As will be seen in the next chapter, these newly formed PML bodies assume the same locus-PML associations seen in this chapter. Likewise, Eskiw *et al* showed that reformation of PML bodies (after heat shock fission of the bodies) occurs by fusion with the parent PML bodies at fixed positions, suggesting that PML bodies are located at preferred sites (Eskiw *et al.*, 2003).

Can locus-PML associations be also formed by the positioning and mobility of the **genomic loci**? As mentioned, the positions of chromosome territories are generally considered to be static in each cell cycle (Edelmann *et al.*, 2001). However, gene-PML associations may also be modified by mobility of genes. Live cell studies by (Chubb *et al.*, 2002), revealed that inserts of transfected lac operator repeats exhibit limited mobility of up to several micrometres. This may be sufficient to generate differences of statistical significance in the locus-PML associations. However, in the short term, such movements appear to be Brownian in nature. It has also been shown that loci can move in a specific direction, that is, towards the interior of a cell (Tumbar and Belmont, 2001). Furthermore, work by Volpi *et al* in our laboratory showed that the position of

the MHC region in relation to the chromosome territory was altered in interferon-treated cells, resulting in more MHC signals outside the chromosomal domain visualised by chromosome paint, even after only 10 minutes of interferon treatment (Volpi *et al.*, 2000). This strongly suggests that regions of chromatin can move in response to acute stimuli, although these movements may simply indicate a less condensed chromatin structure, possibly with loss of matrix attachments or gain of new attachments. Directed movements of chromatin towards an actual nuclear structure have not been shown. (See Section 1.2.1.3 for review.)

As already discussed, I have found that locus positioning external to a chromosome territory was not directed towards a PML body. However, genes that were more likely to be found in a peripheral or external conformation were also those more likely to be associated with PML bodies (but not necessarily at the same time). This suggests that the two observations may still be related. For example, both phenomena may be independently related to gene activation. Consistent with this, it has been shown that local gene density and transcriptional activity also affect the external localisation of loci (Mahy *et al.*, 2002a). Alternatively, it may be that a PML body near an active gene may, by its effect on transcription or regulatory factors, influence that degree of chromatin (de)condensation, and thus the probability of external signals. This may explain why for loci with less PML association, generally fewer external loci are observed while for loci with higher PML association, the number of external signals is more variable. This hypothesis is speculative, but consistent with the presence of chromosome remodelling proteins, such as CBP, in PML bodies (see Section 1.3.5 and later in Chapter 4, Section 4.3).

3.3.2 Possible causes of genome-PML association

There are several possible explanations for what may cause a PML body to associate with transcriptionally active regions. PML bodies could be involved transcription in general or in the transcription of a specific subset of genes. Given the limited number of PML bodies (10-30) in a cell compared with sites of transcription (several thousands), this may imply that PML affects only a subset of genes. This would certainly be true if PML bodies were directly interacting with active transcription in these genes, as proposed for other domains, such as SC-35 domains or Cajal bodies (Frey and Matera, 1995; Smith *et al.*, 1999).

However, I have shown that PML bodies associate *indirectly* with transcription sites, and it may be proposed that their role in transcription is likewise indirect. They may thus be distributed such that each PML body interacts with a domain of dense nearby transcription foci. An attractive, but unproven model would be that PML bodies are arranged such that their surroundings form "spheres of influence", which cover most, if not all, transcriptional activity within a nucleus (e.g. within a radius of 1-2 μm). This would mean the PML bodies themselves are distributed such that each PML body affects a "territory" of transcription foci. The analysis of association of PML bodies with general transcriptional activity has been studied by Kiesslich *et al*, using fluorouridine labelled nascent RNA (Kiesslich *et al.*, 2002). In this study, however, only transcription foci in direct contact with PML bodies were counted, and it was found that approximately 30% of PML bodies were associated directly with these foci. This study is does not in itself support my hypothesis. Firstly, a labelled focus only suggests a region that is being transcribed during the time of fixation. This does not imply that this region is highly transcribed. Secondly, to address my hypothesis would require analysing the density of transcription foci within a certain radial distance of each PML body. I have also performed experiments labelling transcription foci by flurouridine (results not shown, but a similar example is given in Figure 5.7.C). Using histogram analysis of the density of transcription foci signals around PML bodies, I have noticed that there appears to be increased number of foci around some PML bodies (data not shown). Formal assessment of this relationship involves much more complex mathematical modelling than that of the mmd measurements, and was thought not feasible.

The question still remains: what is the molecular basis for such an indirect association between PML bodies and transcription site? One possibility is that PML form at sites of high concentration of transcription factors or products. Evidence for this arises from the presence for RNA polymerase II or nascent RNA visualised by fluorouridine, in or around PML bodies, observed by both light and electron microscopy (Boisvert *et al.*, 2000; Kiesslich *et al.*, 2002; LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000). PML bodies also recruit CBP, a transcriptional co-activator with histone acetyltransferase activity involved in multiple pathways (Boisvert *et al.*, 2001; LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000). In addition, many diverse transcription factors or mediators have been found in PML bodies. PML bodies may therefore function as coordinating, depot or degradation structures, to sequester or release transcription factors, as discussed in

Chapter 1. This model is consistent with the function of PML bodies as a depot (Negorev and Maul, 2001).

In addition to soluble factors, transcriptionally active genes have chromatin properties that may favour an association with PML bodies. As mentioned, PML bodies associate with the active X chromosome, in contrast with the inactive X chromosome. Histone modification and DNA methylation are properties that are known to distinguish these two chromosomes, rather than actual gene content (Jeppesen *et al.*, 1992; Norris *et al.*, 1991). A spatial association was already been noted between PML bodies and acetylated histone domains, but not formally characterised or measured (Boisvert *et al.*, 2000). In addition, other structural chromatin variables that I have not explored include Alu sites and GC-rich isochores. However, because of the correlation with gene-dense regions, such conformations will undoubtedly show some degree of association with PML bodies. These, and other chromatin structures in turn contribute to the non-uniformity within chromosome territories (Verschure *et al.*, 1999). Areas of denser chromatin have been shown to be regions of transcription in the interior of the nucleus. Conversely, areas of heterochromatin exist in the nucleus, containing silenced genes. Ultimately, it may be that PML bodies form preferentially in euchromatic regions.

In either case, whether PML bodies localise due to chromatin structure or soluble transcription factors, the question of the 'chicken or egg' arises. Due these various factors pre-exist and allow the PML bodies to form/aggregate, or does the presence of PML bodies alter the environment around them? Such questions are important, but will be difficult to answer.

Thus, to summarise, PML bodies associate with transcriptionally active genomic regions. This further strengthens the evidence that (a) nuclear organisation exists, in particular between protein structures and chromatin, and (b) PML bodies are related, albeit indirectly, to transcription. The next chapter will attempt to determine whether PML bodies play a role in transcription, in particular whether the presence of PML bodies affect transcription.

Chapter 4: The role of PML bodies in transcription

4.1 Introduction

A major question arising from the results in the previous chapter is whether PML bodies affect transcription of the genomic regions with which they are spatially associated. As reviewed in Chapter 1, previous reports have already suggested involvement of PML bodies with the general transcription machinery as well as with multiple pathways. Furthermore, the genes or pathways proposed to involve PML bodies appear to be diverse and not to bear functional relationship to each other.

4.1.1 PML bodies and general transcription factors

An in-depth review of the general transcription machinery has been published (Orphanides and Reinberg, 2002). Briefly, transcriptional activation starts with the binding of the pre-initiation complex to the promoter of a gene. This pre-initiation complex contains the RNA pol IIa (hypophosphorylated) enzyme and general transcription factors TFII-A to -H. Other complexes which mediate the various regulatory signals are then recruited, such as Srb-mediator and Swi-Snf, and the transcriptional coactivator protein CBP. Some of these complexes, including CBP, have chromatin modification activities (Cho *et al.*, 1998a). Activation of the complex results in phosphorylation of the carboxyl tail of the polymerase (RNA pol IIo), which dissociates from the transcription complex and recruits elongation factors.

A possible role of PML bodies in RNA pol II mediated transcription has already been discussed in Section 1.3.7.1. In particular, a subset of PML bodies associates with RNA pol IIa and with CBP (von Mikecz *et al.*, 2000). This study suggested that PML bodies might be involved in supplying or recycling transcription complexes. However, it has recently been noted that the pattern of RNA pol II is dependent on the fixation techniques, and artefactual distributions may therefore occur (Guillot *et al.*, 2004). Furthermore, Grande *et al* showed that other general transcription factors, such as TFIIH and E2F, as well as glucocorticoid receptor (GR) do not colocalise with PML bodies (Grande *et al.*, 1996). In another study, CBP has been shown to colocalise with PML bodies only when tested with antibodies directed specifically at the amino terminal end of CBP (Doucas *et al.*, 1999). The interaction of PML bodies with the general transcriptional machinery thus remains controversial.

4.1.2 PML bodies, the MHC and the interferon pathway

It has been suggested that PML bodies mediate or facilitate the interferon pathway and the transcriptional downstream events. As mentioned in Chapter 1, the *PML* gene is a target of both type I (IFNα and -β) and II (IFNγ) interferons, and stimulation by them leads to an increase in the number of PML bodies (Regad and Chelbi-Alix, 2001). This upregulation of PML by interferons is thought to mediate the growth suppression and apoptotic effects of interferons in some cell lines (Chawla-Sarkar *et al.*, 2003; Vannucchi *et al.*, 2000). A role of PML bodies in mediating IFN-induced transcription has also been suggested by several experiments. For example, IFNγ treatment of cells results in an increased association of PML bodies with transcription foci (labelled with fluorouridine) (Kiesslich *et al.*, 2002), while IFNα treatment results in recruitment of DNA helicase II and RNA polymerase II into PML bodies (Fuchsova *et al.*, 2002).

PML bodies are also implicated directly in the expression of the MHC genes. The demonstration of a spatial association between the MHC and PML bodies has already been discussed (Shiels *et al.*, 2001). Transfection of PML has been shown to induce expression of the *HLA-A, -B,* and *–C* genes and the *LMP-2* and *–7* genes in a mouse tumour cell line deficient in their expression (Zheng *et al.*, 1998). MHC class I expression (heavy chain and β2-microglobulin) has been shown to be restored in some (but not all) human lung cancer cell lines by the introduction of PML protein (Chang *et al.*, 2002). Furthermore, loss or disruption of PML expression has been associated with downregulation of MHC class I expression in both prostate and colon cancer tissue specimens (Cabrera *et al.*, 2004; Zhang *et al.*, 2003).

However, there is also evidence that PML bodies are not necessarily involved in expression of the MHC genes. APL cell lines as well as PML -/- cells retain normal MHC class I expression (Larghero *et al.*, 1999; Ruggero *et al.*, 2000). More recently, RNAi knockdown studies showed that reducing PML expression did not alter MHC class I expression, at least in human cell lines (Bruno *et al.*, 2003). These studies suggest differential effects based on either specific cell types or species.

The regression analysis described in Chapter 3 suggested that the association of PML bodies with a genomic region correlates with the general transcriptional activity in the region, rather than with the activity of one specific gene. However, several properties of the genes within the regions studied are worth mentioning again. Genes that are

paralogous to the MHC are not necessarily associated with PML bodies. This may be due the fact that some of these genes have divergent functions. Instead, several genes with immune function have the same degree of association as the MHC. This includes some of the proteasome subunit genes that are controlled by IFNγ (TAP/LMP, *PSMB5, PSMB7, PSMB10* and *PSME1/2*). I noted that some of the other genes that showed a high degree of association with PML bodies are also IFNγ-responsive. These included a set of genes encoding structural proteins, and which had been tested elsewhere for association with SC-35 domains (Smith *et al.*, 1999; Xing *et al.*, 1995). Of these, the *COL1A1* and *LMNA* genes are downregulated by IFNγ (Boehm *et al.*, 1997).

It must be mentioned that many of the immune-related genes tested lie in gene-rich regions and may be clustered to facilitate coordinated expression. Examples include the KIR/ILT gene cluster on chromosome 19 (Wilson *et al.*, 2000). It was therefore not possible to determine, using our statistical model and regression analysis, whether the association is related to the presence of functionally related gene clusters, rather than the level of transcription in the region and the gene density. Furthermore, many genes with non-immune functions also lie in gene-rich regions containing immune genes and vice versa. Examples are the *NOTCH1* and *ABC2* genes which, although not directly immune related, lie in chromosome band 9q34 containing immune-related genes, including the proteasome subunit *PSMB7*. Therefore, analysis of individual genes does not take into account the other genes around them. Nevertheless, the data thus far demonstrate that while PML bodies associate with a large group of immune-related genes, they are also strongly associated with other genes having disparate functions.

In view of the previous and current evidence linking PML bodies to interferons and the MHC, it is important to understand further the signalling pathway and downstream events induced by interferon, and in particular IFNγ.

4.1.3 The effects of IFNγ in the cell

Interferons are cytokines involved in cellular immunity. Type I interferons (IFNα and β) have specific anti-viral roles, being secreted by virus-infected cells. In contrast, the type II member of the interferon family, IFNγ, is secreted by T-cells and natural-killer (NK) cells and acts more as a general cytokine, regulating over 200 genes (reviewed in Boehm *et al.*, 1997). While type I and II interferons generally induce different sets of

genes, there is some overlap (Der *et al.*, 1998). Furthermore, the type I and II interferons can act synergistically (Gao *et al.*, 1993).

The secretion of IFNγ by T- and NK-cells is regulated by other cytokines (e.g. TNFα and IL-12), as well as by itself, forming an autoregulation loop (Hardy and Sawada, 1989). IFNγ acts by binding to a specific cell surface receptor (IFNγR) comprising two subunits, which is ubiquitously, but not uniformly expressed on all nucleated cells. Binding of IFNγ to IFNγR causes the subunits to dimerise. This results in the phosphorylation of two tyrosine kinases (the Janus kinases, JAK1 and JAK2) (Darnell *et al.*, 1994). These mediate tyrosine phosphorylation and homodimerisation of the transcription factor STAT1 (signal transducer and activator of transcription-1) (Lee and Benveniste, 1996), which then binds the gamma-activation sequence (GAS) of IFNγ responsive genes. The JAK/STAT pathway is also employed by other members of the cytokine receptor superfamily to which the IFNγR belongs, but variations in the STATs activated lead to different effects. IFNγ and the type I interferons are also thought to act via different pathways (reviewed in Parmar and Platanias, 2003).

Some of the primary response genes encode transcription factors, which subsequently mediate secondary responses, which are delayed, due to the necessary protein synthesis. Such mediators include the IRF (interferon-responsive factors) family. IFNγ induction of the HLA class I and II genes are two such secondary responses (Figure 4.1.A).

4.1.4 Control of HLA Class I gene expression

The various upstream sequences of the HLA class I genes are shown in Figure 4.1.B (reviewed by van den Elsen *et al.*, 1998). These genes can be regulated by a variety of cytokines including TNFα (via NF-kB) and IFN types I and II. Upregulation of their expression by IFNγ occurs via the interferon-stimulated response element (ISRE) in the IFN consensus sequence (ICS) site, which binds IRF-1, IRF-2, IRF-8 (ICS-binding protein) and IRF-9/ISGF3γ/p48. IRF-1 and IRF-2 are both induced by the primary JAK/STAT pathway (Harada *et al.*, 1989). There are no STAT1 binding sites for these genes. However, STAT1 and -2 associate with ISGF3γ to form the ISGF3 complex which also binds to ISRE (Bluyssen *et al.*, 1996).

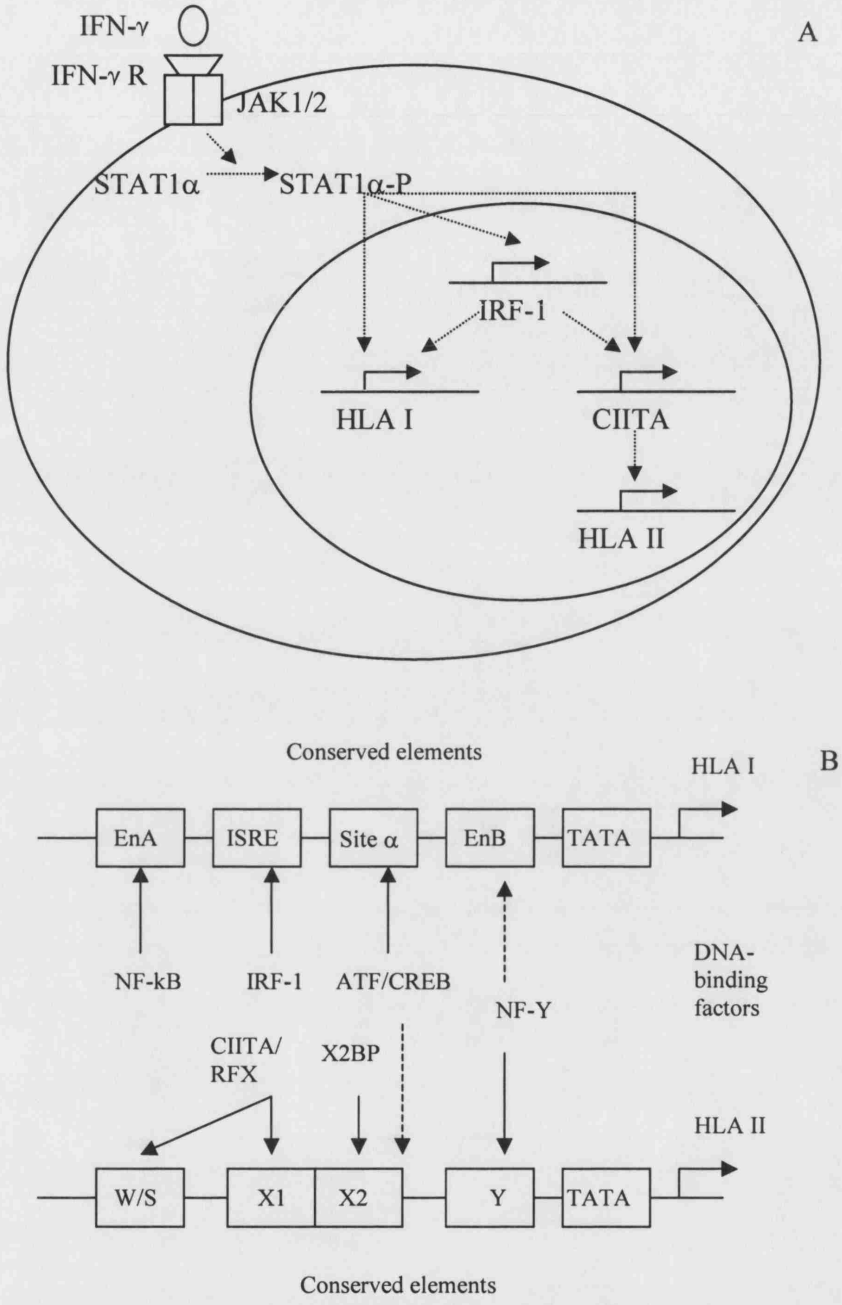

Figure 4.1. The effects of IFNγ on the MHC expression.
A: Diagram of the interferon mediated pathway for MHC expression.
B: The promoter regions and binding factors of HLA class I and II genes. CIITA is a key factor in HLA class II expression, but does not bind DNA directly. Solid arrows indicate the proven transcription factors. Dotted arrows indicated the proposed factors.

HLA class I genes are also induced by TNFα acting via NF-kB, which binds to two kB binding elements in the enhancer A site (Gobin *et al.*, 1998). The enhancer A and ISRE elements mediate synergy between the activities of TNFα and IFNγ (Johnson and Pober, 1994). Furthermore, NF-kB and IRF-1 have been shown to interact physically (Drew *et al.*, 1995).

The upregulation of HLA class I expression as detected by the increase in mRNA levels is seen 6 to 24h following IFNγ stimulation (Min *et al.*, 1996). In that study, *TAP1* gene expression (which is required for antigen presentation by HLA class I molecules on the cell surface) was found to be upregulated within 2h of IFNγ treatment. This is likely to be due to the fact that the *TAP1* promoter has sequences corresponding to both GAS and ISRE binding sites, thus allowing upregulation by STAT1.

4.1.5 Control of HLA Class II Gene Expression

As mentioned, HLA class II cell surface expression is normally limited to antigen presenting cells, where their expression can be upregulated by various cytokines, such as TNFα, interferons and interleukins (reviewed by Ting and Trowsdale, 2002; van den Elsen *et al.*, 1998). However, in nearly all other cell types, HLA class II expression can be induced by cytokines including IFNγ. Both basal and inducible HLA class II expression are dependent on a number of highly conserved promoter sequences, as shown in Figure 4.1.B.

The classical and accessory genes involved in the class II antigen-presentation machinery are regulated by the class II transactivator (CIITA) (Steimle *et al.*, 1994; Zhou and Glimcher, 1995). CIITA is a protein that does not bind DNA directly, but acts via the RFX complex (Riley *et al.*, 1995). CIITA expression is usually restricted to constitutively MHC class II-positive cells, but it is induced by IFNγ in other cell types (Chang *et al.*, 1994). Loss of CIITA and some members of the RFX complex accounts for the various subgroups of the Bare Lymphocyte Syndrome (BLS). Patients with this condition are unable to express class II molecules on the B- and T-cells, either constitutively or after IFNγ treatment (Steimle *et al.*, 1993).

Depending on the cell type, CIITA mRNA appears at approximately 1 to 2h after IFNγ stimulation (Chang *et al.*, 1994; Lee and Benveniste, 1996; Steimle *et al.*, 1994). In

contrast, MHC class II mRNA is only detected 8 to 12h after IFNγ stimulation. The mRNA levels then peak at 24 to 48h and diminish after 72h (Bottger *et al.*, 1988; Vidovic *et al.*, 1990).

Therefore, IFNγ-mediated transcription is a complex process involving a variety of mediators, as illustrated by upregulation of the MHC class I and II genes. In addition, basal transcription is itself a multistep complex process involving elaborate transcriptional machinery. As discussed, both processes have potential interactions with PML bodies.

In the work described in this chapter, the aim was to analyse the effect of PML bodies on transcription. I therefore set out to determine whether PML bodies influence the transcription of genes with which they are spatially associated. The rationale was that if PML bodies affect the activity of the general transcription machinery, they are more likely to influence the transcription of nearby genes. Furthermore, since many of these are regulated by interferons, I also explored whether PML bodies influence transcription of genes that are regulated by IFNγ.

4.2 Results

4.2.1 PML bodies and basal transcription

To examine the role of PML bodies in the transcription levels of the genes studied in Chapter 3, two complementary approaches were used: the expression of PML bodies was decreased by RNAi treatment, and increased by transient overexpression of the PML protein.

4.2.1.1 Effect of PML depletion by RNAi on basal transcription

In order to knock down PML expression, I used RNAi treatment by lipofection on MRC5 cells, with siRNA. This involves the transient transfection of 21-mers of double-stranded RNA designed to bind to, and degrade, the target mRNA. Three different siRNAs were initially designed against PML-IV (PML-3) mRNA as per guidelines from www.qiagen.com, but these did not result in effective suppression of PML protein levels. Subsequently, an siRNA targeting exon 2 of PML mRNA and shown to be effective in knockdown of all isoforms of PML protein (aa 398-418) was found (Bruno *et al.*, 2003). The transfection conditions were optimised (see Section 2.1.5). It was

found that most important factor for maximum knockdown of PML protein was incubation of the cells for three or more days following transfection. This may be due to the half-life of the PML protein. MRC5 cells were thus transfected with siRNA, and separate populations of cells transfected with non-silencing siRNA (obtained commercially from Qiagen) and untreated cells were used as controls.

Using immunofluorescence, I found that RNAi treatment resulted in 52% of cells having no PML bodies, with the remainder having approximately half the normal number of PML bodies. The mean number of PML bodies in these RNAi treated cells was 6.7 compared with 13.1 in untreated cells and 15.4 in non-silencing RNAi treated cells (see Figures 4.2.A and 4.2.B). That is, there was no change in the number of PML bodies in the non-silencing RNAi treated cells. Using immunoblotting assays, I found the level of PML protein to be reduced by 60-70% (Figure 4.2.C). Bruno *et al* described a knockdown effect in more than 90% of cells. This apparent discrepancy may be due to the MRC5 cells being less 'transfectable'. Nevertheless, I expected this reduction in PML protein to allow me to determine whether PML bodies affect transcription of genes that are normally closely associated with them.

Semi-quantitative RT-PCR for the 49 genes studied in Section 3.2.5 (i.e. excluding the centromeric regions) was performed first. PCR primers were designed for the full-length cDNAs, to give 200-300bp products which cross exon-exon boundaries where possible. The intensities of the PCR products were measured after correction for the transcription level of *ACTB*, a housekeeping gene. My RT-PCR data showed changes to some of the genes even in non-silencing RNAi treated cells compared with untreated cells. I therefore decided that comparisons between PML RNAi and non-silencing RNAi treated cells were more likely to reflect the specific effects of PML knockdown. Using these comparisons, the transcription levels of genes that are normally closely associated with PML bodies were not found to be more substantially affected than those of genes which are less closely associated with PML bodies. These results were reproduced in three different experiments (Figure 4.3A).

Since the semi-quantitative measurement of gene expression is prone to errors, the results were confirmed using real-time RT-PCR on 12 genes chosen to represent the different levels of association with PML bodies. For this, additional PCR primers were designed against each full-length cDNA to give products of approximately 100bp. PCR

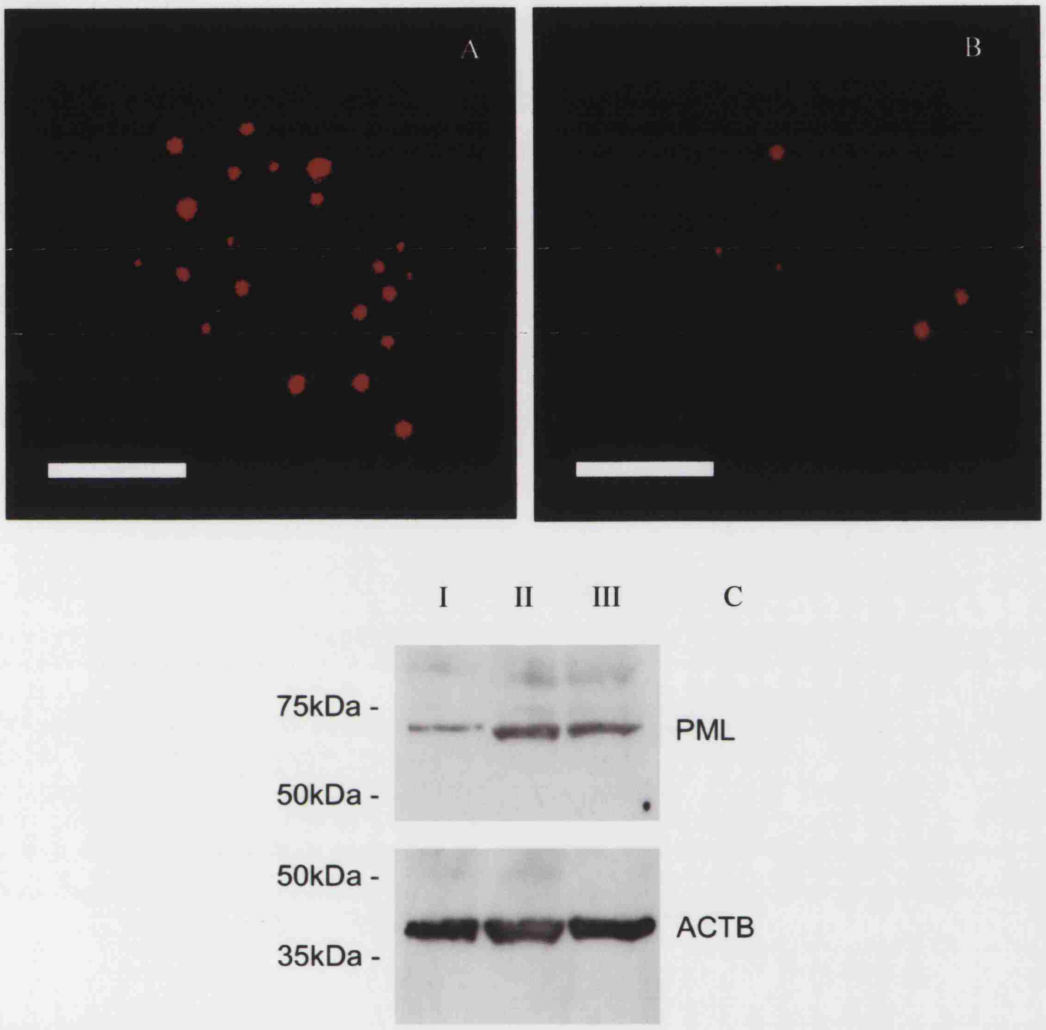

Figure 4.2. The effect of knock-down of PML protein and PML bodies.
A and B: MRC5 cells showing typical PML body distribution in normal (A) and PML siRNA-treated (B) cells. The solid line represents 5 micrometres.
C: Western blots showing PML and b-actin protein levels in (I) PML RNAi cells, (II) non-silencing RNAi treated cells, (III) untreated cells.

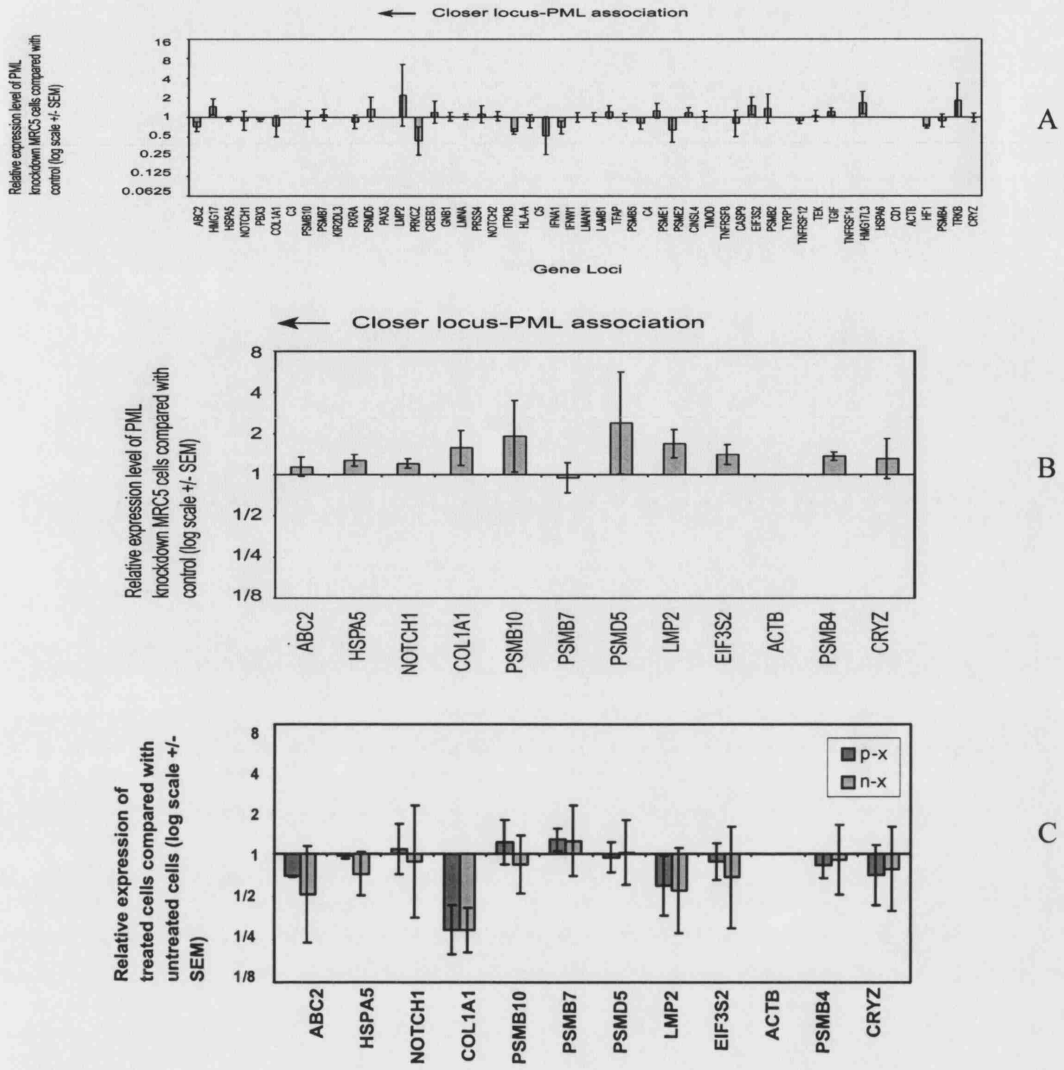

Figure 4.3. The effect of knock-down of PML bodies on basal transcription levels.
A: Graph showing the change in transcription levels of genes in cells treated with PML RNAi, measured by RT- PCR, as compared with cells treated with non-silencing siRNA. 49 genes studied by immunoFISH were analysed and arranged horizontally in increasing mmd-locus values. The vertical axis represents the ratio of transcription level of a gene in RNAi treated cells, to that of the control group (=1), on a $\log_2$ scale. Therefore a change above the line means an increase in expression of a gene, while a change below the line means a decrease in expression, compared to the control group.
B: Graph showing the change in transcription levels of genes in cells treated with PML RNAi, measured by real-time PCR, as compared with cells treated with non-silencing siRNA. Genes studied were arranged in increasing mmd-locus values. Horizontal and vertical axes are scaled as in A.
C: Graph showing the change in transcription levels of genes in cells treated with PML RNAi (P-X) or control RNAi (N-X), compared with untreated cells.

reactions were run in a PCR mix containing SYBR green fluorescent dye. The amount of PCR product was measured during each cycle of the reaction, and the number of cycles required for the SYBR green intensity to reach a calculated threshold (Ct) value was measured. The cut-off value was set manually to the exponential phase of the PCR reaction. Each gene was analysed in duplicate in each sample, and corrected for *ACTB* expression. Whole experiments were repeated at least twice.

Once again, the genes which were more closely associated with PML bodies (*ABC2*, *NOTCH1* and *HMG17*) showed no obvious changes in transcription when compared with those less associated (*PSMB2*, *CRYZ* and *EIF3S2*) (Figure 4.3.B). Thus, it appears that proximity to PML bodies does not influence the basal transcription of these genes. As with the semi-quantitative RT-PCR, non-specific changes were noted when both PML RNAi and non-silencing RNAi samples were compared with the untreated cells (Figure 4.3.C). It has been noted that RNAi treatment can result in upregulation of interferon-responsive genes, but this is specific only to short-hairpin RNA transfections using a plasmid vector, rather than siRNA transfections using liposomal methods (Bridge *et al.*, 2003).

4.2.1.2 Effect of PML overexpression on basal transcription

Many previous reports have shown transcriptional changes of specific genes using co-expression of PML and a specific transcription factor. I therefore decided that overexpression of PML was also required to determine whether PML bodies play a role in basal transcription. I transiently overexpressed full-length PML isoform IV (previously PML-3) cDNA in a constitutively expressed vector in MRC5 cells for three days. As controls, cells transfected with a similar vector expressing lacZa protein (provided in the transfection kit) as well as untreated cells were used.

Firstly, the level of PML protein in transfected cells was compared with untransfected cells. Using Western blotting, it was found that both the PML vector and control vector resulted in increased PML levels in the cell (Figure 4.4.A). This was confirmed using immunofluorescence, where both transfections resulted in increased numbers and sizes of PML bodies (Figure 4.4.B, C and D). As discussed in Chapter 1, PML bodies have been noted to form at sites of foreign protein (Tsukamoto *et al.*, 2000), including proteins associated with the lac operator/repressor complex. It is therefore likely that the transfection event itself results in upregulation of PML.

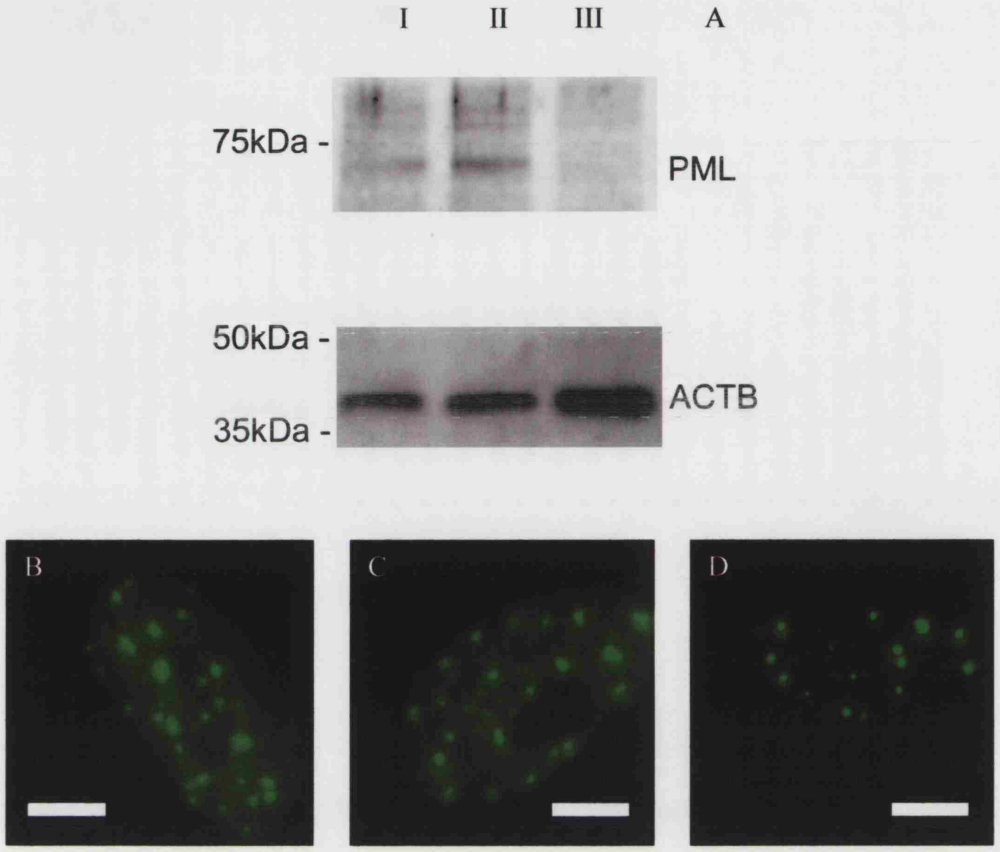

Figure 4.4. The effect of overexpression of PML protein and PML bodies.
A: Western blots showing PML and b-actin protein levels in (I) PML overexpressed cells, (II) control vector treated cells, (III) untreated cells. When corrected for the ACTB intensities, the PML quantity in overexpressed cells (a) was 8.0x of untreated cells, and in control transfection (b) was 6.5x of untreated cells.
B: MRC5 cell transfected with PML-containing vector.
C: MRC5 cell transfected with control vector showing increased PML expression(green).
D: MRC5 cell showing typical PML body distribution (green).
The solid line represents 5 micrometres.

Real-time PCR was performed again on the 12 genes mentioned in the previous section. No obvious trend was noted when the expression of these genes was compared between the PML and control transfection studies (Figure 4.5.A, three samples were tested and averaged). Interestingly, increased expression was found for the *LMP2* gene, and to a lesser extent, the *PSMB10* gene, in both the PML and control vector transfected cells, compared with untransfected cells (Figure 4.5.B, please note the different scale on the y-axis). This suggests that the MHC antigen presentation machinery may be activated by the transfection event itself. In fact, transfection of DNA fragments has been shown previously to upregulate the MHC antigen-presentation machinery (Suzuki *et al.*, 1999).

I thus concluded that overexpression studies are unlikely to be meaningful in the context of PML proteins and immune responses, as the transfection event leads to non-specific effects on both MHC and PML expression.

4.2.1.3 Genomic associations of newly formed PML bodies in NB4 cells

NB4 is an APL cell line derived from a leukaemia patient. ATRA treatment of NB4 cells mirrors that of leukaemic cells in patients, in that it results in the formation of structurally 'normal' PML bodies, replacing the microspeckled aggregates of the PML-RARα fusion product (Dyck *et al.*, 1994) (Figure 4.6.A and 4.6.B). Thus, the spatial organisation of PML bodies can be examined as they form in the nucleus.

NB4 cells were grown in suspension and treated with ATRA at a final concentration of 1 μM for 24-48 hours (Lee *et al.*, 2002). ImmunoFISH experiments were performed to detect PML bodies, the TAP/LMP locus, and either the 6p24 locus or the chromosome 6 centromere (Figure 4.6.B, Appendix C.6). I found that when NB4 cells were treated with ATRA, newly formed PML bodies were positioned statistically closer to the TAP/LMP locus than the 6p24 locus or the chromosome 6 centromere (Figure 4.6.C). These experiments clearly show that PML bodies form preferentially in specific nuclear regions or environments in ATRA-treated NB4 cells as well as MRC5 fibroblasts (in the previous chapter). The question, therefore, was whether in these ATRA-treated NB4 cells, PML bodies form in response to the transcription state pre-existing in the MHC, or whether PML bodies alter transcription in this region.

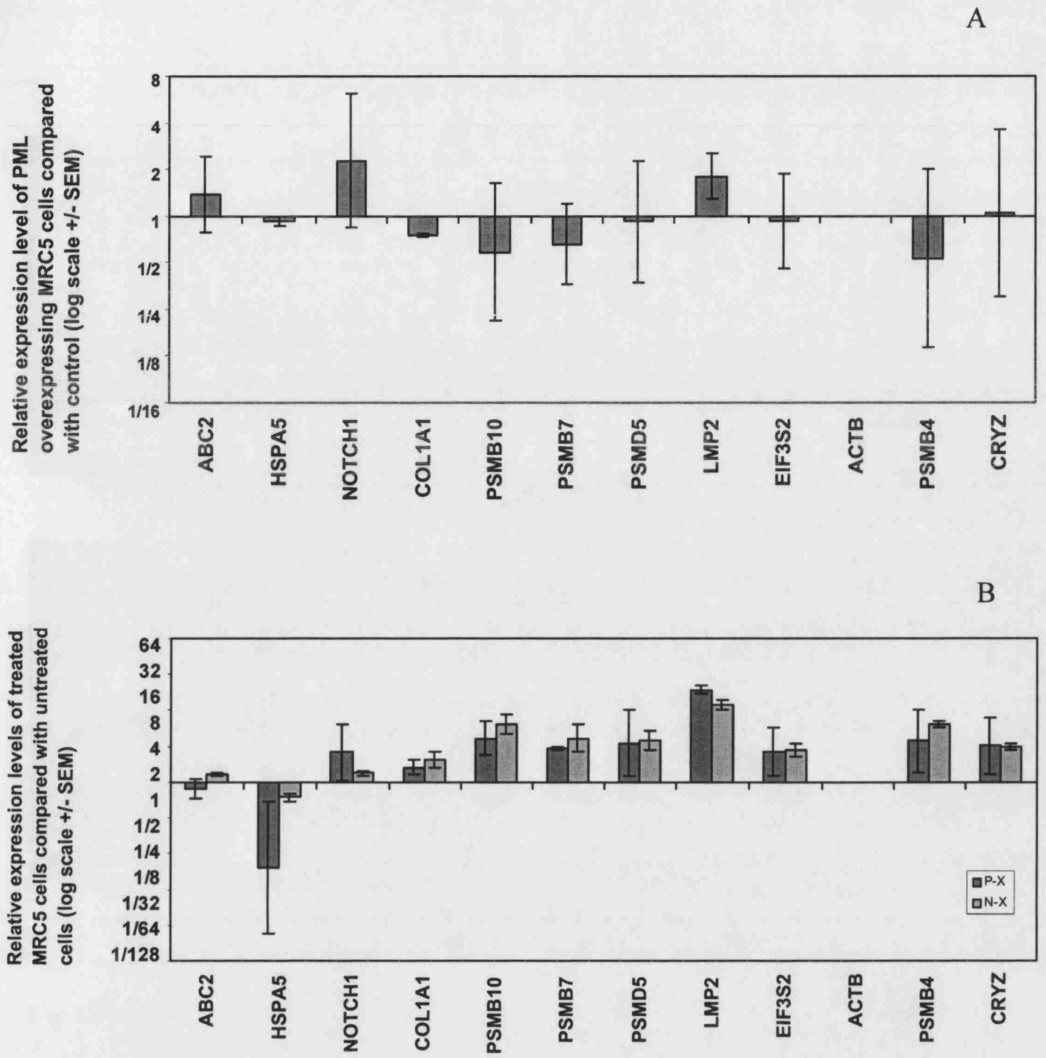

Figure 4.5. The effects of overexpression of PML bodies on basal transcription levels.

A: Graph showing the changes in transcription levels of genes in MRC5 cells transfected with an expression vector for PML, compared to cells transfected with a control vector, measured by real-time PCR. Loci studied were arranged in increasing mmd-locus values. Horizontal and vertical axes are scaled as in Figure 4.3.

B: Graph showing the change in transcription levels of genes in cells treated with PML transfection (P-X) or control transfection (N-X), compared with untreated cells.

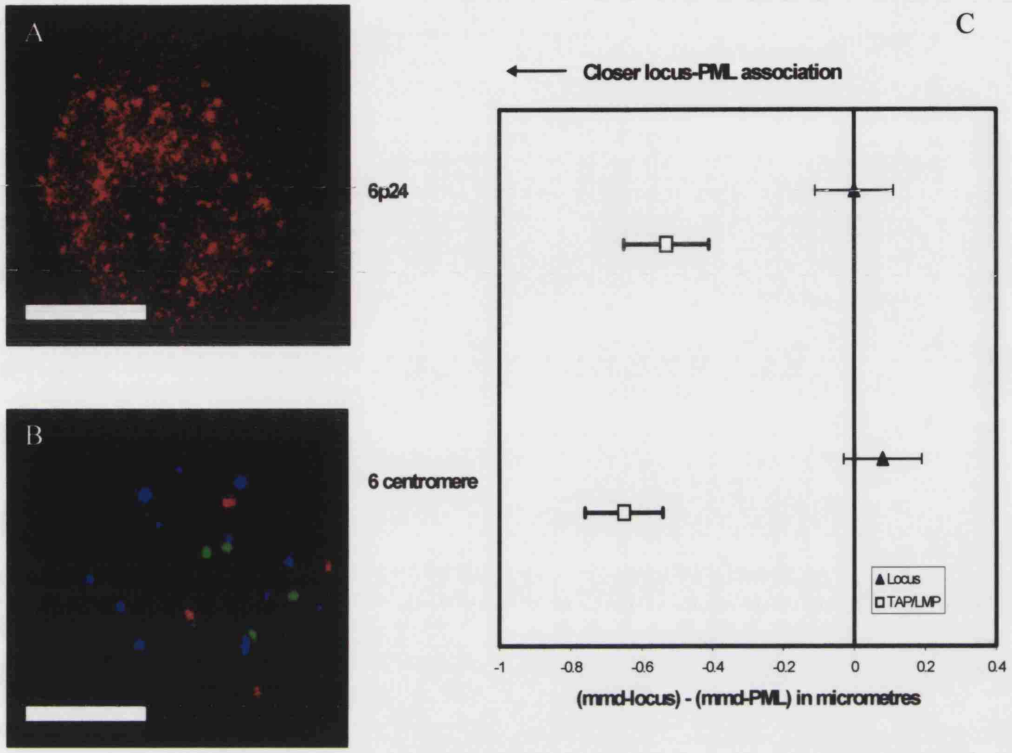

Figure 4.6. The formation of new PML bodies in NB4 cells.
A and B: NB4 cells showing the microspeckled pattern of PML protein (red) prior to ATRA treatment (A), and the formation of PML bodies (blue) after differentiation (B). (B) also shows four TAP/LMP loci (green) and 6p24 loci (red) in the treated cells (NB4 cells have a hypotetraploid karyotype). The solid line represents 5 micrometres.
C: Locus-PML distances for different loci were compared following ATRA treatment, with error bars showing 1 standard error. Paired T-tests were performed between the TAP/LMP and either the 6p24 or chromosome 6 centromere loci. TAP/LMP was significantly more associated with the newly formed PML bodies than the other two loci.

4.2.1.4 Effect of ATRA treatment on transcription in NB4 cells

Previous studies showed alterations in the expression of multiple genes in NB4 acute promyelocytic leukaemia cells treated with ATRA (Lee *et al.*, 2002). I therefore wished to investigate whether ATRA treatment of NB4 cells induced alterations in the transcription levels of the 49 genes studied. Semi-quantitative RT-PCR analysis suggested that some of the genes that are closely associated with PML bodies are more likely to be downregulated by ATRA treatment (Figure 4.7.A). Real-time RT-PCR was then performed for the subset of 12 genes as in previous sections, but did not corroborate the initial results. In particular, the PML body-associated genes *NOTCH1* and *HMG17* were not found to be downregulated by ATRA treatment using real-time RT-PCR, in contrast to the semi-quantitative data, although the *ABC2* gene was downregulated in both experiments (Figure 4.7.B).

Potentially more interestingly, it was noted in both the semi-quantitative and real-time results that the *LMP2* gene was upregulated by ATRA treatment of the NB4 cells. Since the experiments described in previous section showed that newly formed PML bodies in NB4 cells associated with this locus (compared statistically with the 6p24 and centromeric loci), a link between positioning of the PML bodies and transcription changes within the MHC may exist. However, the MHC, being a gene-rich region, is also recognised as a RIDGE (Caron *et al.*, 2001). This locus therefore contains many genes that may already be actively transcribed. The question, however, still remains as to whether PML bodies form in response to local transcriptional activity (or changes in transcription), rather than being responsible for nearby activity.

It must also be noted that no inverse correlation was observed between the effects of ATRA treatment of NB4 cells and RNAi knockdown experiments in MRC5 cells (Figure 4.8). ATRA treatment of NB4 cells presumably leads to more complex changes in gene expression as a result of induction of myeloid differentiation (Lee *et al.*, 2002), compared with the effect of RNAi knockdown. This may therefore be the case for the *LMP2* gene.

To study if the formation of PML bodies in the vicinity of the MHC locus is related to transcriptional changes in the region, experiments were carried out as described in the next section to determine if IFNγ treatment affects the association of PML bodies with

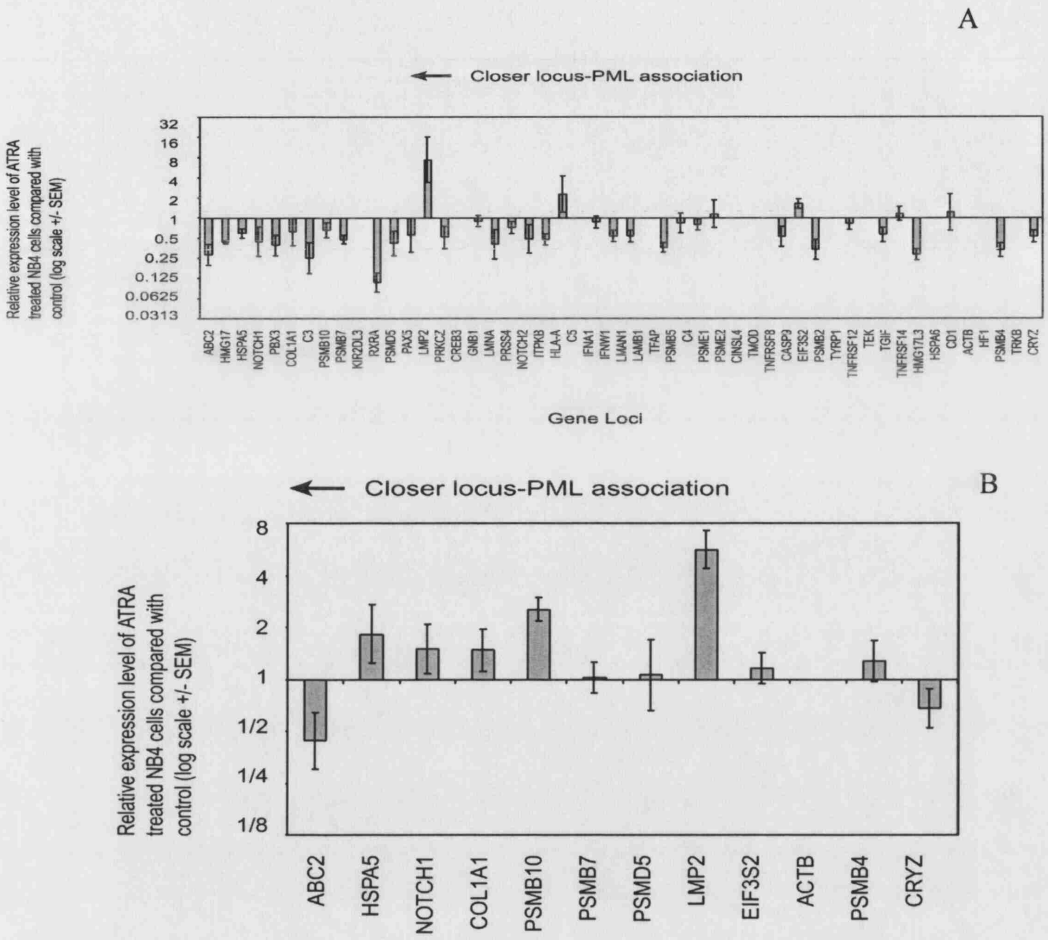

Figure 4.7. The effect of formation of new PML bodies on basal transcription levels.

A: Graph showing the change in transcription levels assessed by semi-quantitative RT-PCR of genes in NB4 cells following treatment with ATRA, compared with untreated cells. 49 loci studied by immunoFISH, were analysed and arranged horizontally in increasing mmd-locus values from analysis in MRC5 cells. The vertical axis represents the ratio of transcription level of a gene in RNAi treated cells to that of the control group (=1), on a $\log_2$ scale.

B: Graph showing the change in transcription levels assessed by real-time PCR of genes in NB4 cells following treatment with ATRA, compared with untreated cells. Horizontal and vertical axes are scaled as in A.

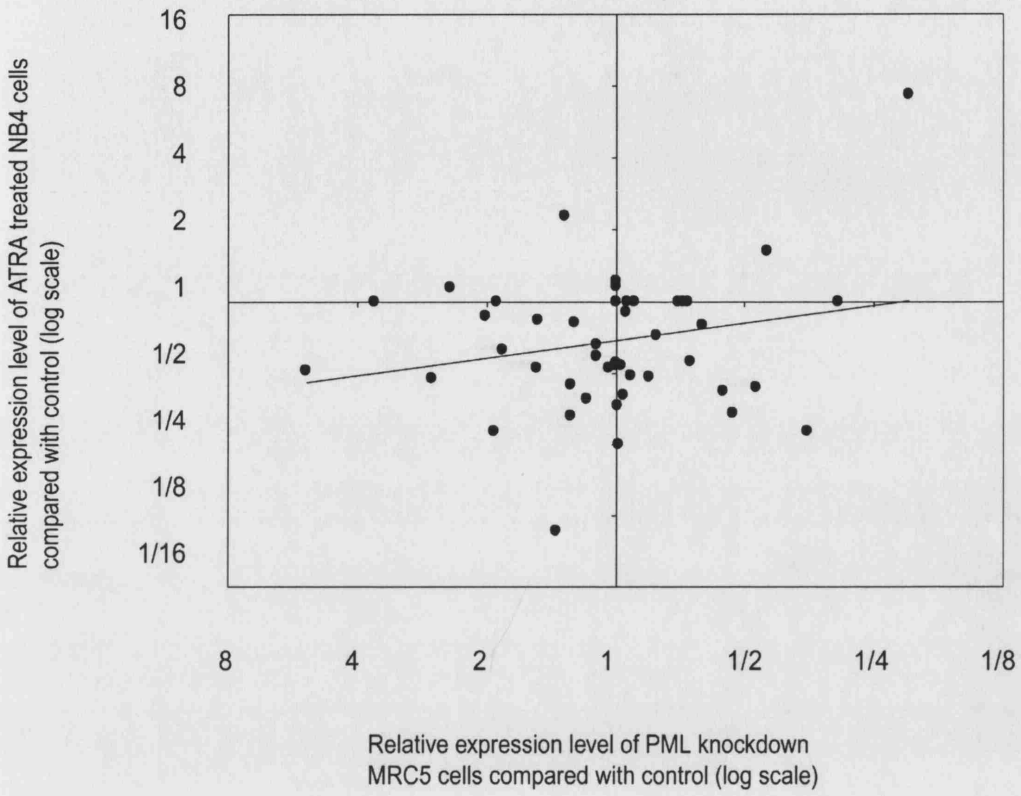

Figure 4.8. Linear regression graph comparing the effect of PML RNAi knock-down in MRC5 cells with ATRA treatment in NB4 cells.
Results from Figure 4.3 (A) (on x axis) were analysed against results from Figure 4.7 (B) (y axis). Each bullet represents a locus. Each graph displays the best line fit for the regression. The statistical results were R^2=0.05, F=2.44, p=0.12.

the MHC. The next section also attempts to address the broader question of whether PML bodies play a role in interferon-mediated transcription.

4.2.2 PML bodies and interferon mediated transcription

4.2.2.1 Effect of IFNγ treatment on locus-PML association

Shiels *et al* showed that treatment of MRC5 cells with IFNγ increased the observed visual association of PML bodies with the TAP/LMP locus. However, this was accompanied by an increase in the size and number of PML bodies, resulting in no statistical increase in the degree of association in interferon-treated cells compared to untreated cells, when corrected for the PML-PML distances. This prompted me to explore further the effect of interferon treatment on PML associations with other loci.

ImmunoFISH experiments were performed as in Chapter 3. Here, the mmd measurements for different loci were compared in IFNγ treated and untreated cells. IFNγ treatment was performed at a concentration of 200 or 500U/ml for 24 hours. I found that for the TAP/LMP locus and two other proteasome subunit genes (*PSMB7* and *PSMB10*), the mmd distance was decreased in treated compared to untreated cells. However, when corrected for the changes in the PML-PML mmd distances, statistical analysis showed no increase in association of each of the three loci with PML bodies in IFNγ-treated compared to untreated cells (Figure 4.9.A, Appendix C.7). Previous studies have shown that IFNγ upregulates expression of the *PSMB10* gene (as well as the *LMP2,7/PSMB8,9* genes), while it downregulates *PSMB7* expression (Griffin *et al.*, 1998). In an immunoFISH experiment where all three loci were detected together with PML bodies, I noted no obvious preference of the three proteasome subunit genes for the same PML body (Figure 4.9.B). Thus a single PML body does not serve to coordinate different genes in concert.

Since all three loci studied were affected by IFNγ and they gave similar results, it was difficult to assess whether there was a functional association between PML bodies and each locus in IFNγ-treated cells. For example, the increased number of PML bodies may actually be important in transcription, and the decrease in mmd distances may reflect a true closer locus-PML association, rather than the corrected measurement. Therefore, as controls, I examined the 6p24 locus and the centromere on chromosome 6, and found that for these two transcriptionally silent regions that are not known to be affected by

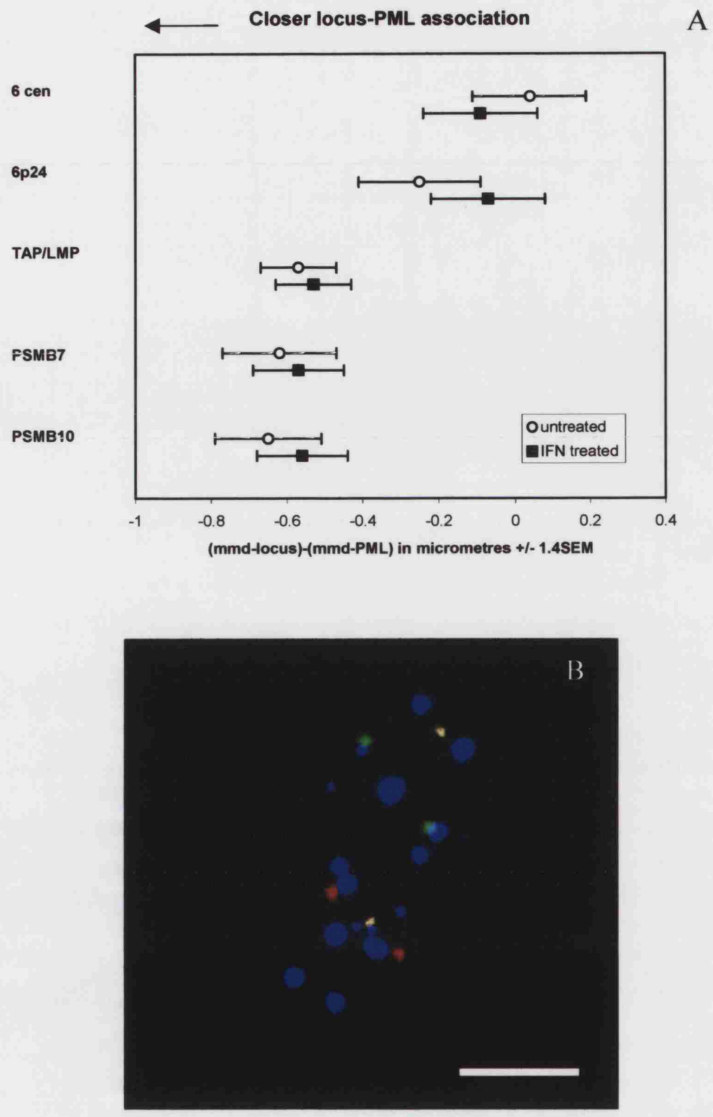

Figure 4.9. Association of PML bodies with loci in IFNγ-treated MRC5 cells.
A: MRC5 fibroblast cells were studied, with respect to their response to IFNγ. Locus-PML distances for different loci were compared between treated and untreated cells. The (mmd-locus) – (mmd-PML) on X-axis represents the distance to which a locus is further or closer to the nearest PML body compared with the PML-PML distance, with error bars showing 1.4 standard errors. Unpaired T-tests were performed between the treated and untreated cells of each cell line. None of these regions showed a statistically significant change in association with PML bodies.
B: MRC5 cell nucleus showing the TAP/LMP locus (green), *PSMB7* locus on chromosome 9 (yellow: combined green and red), *PSMB10* (red) on chromosome 16, and PML bodies (blue). Genes encoding different proteasome subunits do not associate by direct contact with the same PML body. The solid line represents 5 micrometres.

IFNγ, there was also a decreased mmd distance to PML bodies in IFNγ-treated cells. Once again, the differences between treated and untreated cells were not statistically significant for these loci when corrected for PML-PML distances.

These experiments suggest that in IFNγ-treated cells the observation of increased visual associations of all the regions studied relates more to the increase in the number of PML bodies and the volume they occupy within the nucleus. These controls also validate further the advantages of mmd distance measurements, especially when corrected for the PML-PML distances. The inclusion of the two control loci in this study confirms the initial observation regarding the TAP/LMP locus, that interferon treatment, while increasing the number of PML bodies in a nucleus, does not result in a preferential association of PML bodies with specific loci which are upregulated by IFNγ. Thus, the formation of PML bodies in the vicinity of the MHC in NB4 cells (in the previous section) is unlikely due to any upregulation of the *LMP2* gene *per se* by ATRA.

4.2.2.2 Effect of RNAi on the IFNγ-response

To test more directly whether PML bodies are involved in the interferon pathway, I extended the RNAi studies mentioned previously. MRC5 cells were treated for 3 days with PML-silencing siRNA transfection, with non-silencing siRNA transfected cells and untreated cells as controls. Sub-populations of these cells were then treated with IFNγ at 200U/ml for 2, 8 and 24 hours prior to harvesting for RNA extraction. Real-time PCR was performed as before on a subset of genes that were recognised to be IFNγ-responsive (Boehm *et al.*, 1997). These were *LMP2* (*PSMB9*), *HLA-DRA*, *PSMB10* (all upregulated), *PSMB7* and *COL1A1* (both downregulated), with *ACTB* as a control.

As expected, in the untreated MRC5 cells the *LMP2*, *HLA-DRA* and *PSMB10* genes were upregulated by IFNγ from 2 and 8 hours, with mRNA levels reaching a maximum at 24 hours. Expression of these genes was upregulated to 4 to 32 times basal levels. Downregulation of *PMSB7* and *COL1A1* genes, however, was less dramatic, with mRNA decreased only two-fold (i.e. to half) (Figure 4.10). This may be due to the effect of the concentration of IFNγ used, or to the presence of existing mRNA levels of the *PSMB7* and *COL1A1* genes.

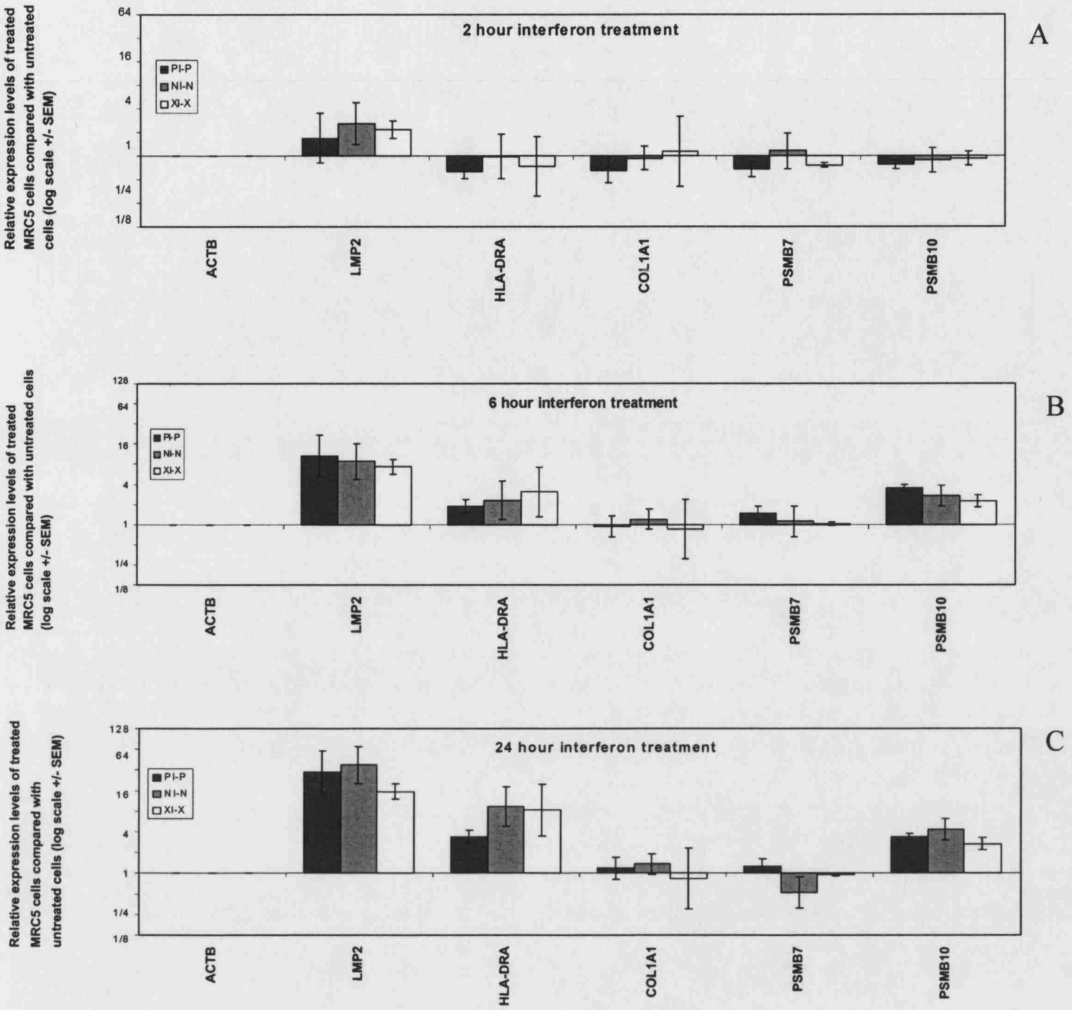

Figure 4.10. The effects of knock-down of PML bodies and IFNγ on transcription levels.

Graphs showing the change in transcription levels of genes in cells treated with PML RNAi, (or controls) followed by IFNγ treatment for 2 (A), 6 (B) or 24h (C). 5 loci known to be interferon responsive were studied as well as the ACTB gene. The vertical axis represents the ratio of transcription level of a gene in IFNγ treated cells, to that of the untreated cells (=1), on a $\log_2$ scale. The effect of IFNγ was compared across the graph for PML RNAi treated cells (expression of gene in IFNγ-treated & PML knockdown cells compared with non-IFNγ-treated but PML knockdown cells, PI-P, dark grey), non-silencing RNAi treated cells (NI-N, light grey), and RNAi untreated cells (XI-X, white). RNAi treatment had no effect on the response of MRC5 cells to IFNγ.

Neither siRNA nor non-silencing RNAi treatment were found to affect the transcriptional response of these genes to IFNγ. This was especially obvious for the genes, *LMP2*, *HLA-DRA* and *PSMB10*, which still showed upregulation of gene expression by IFNγ. The magnitude of increases in mRNA levels was similar to the IFNγ treatment of cells not transfected with PML siRNA.

The results of this section suggest that PML bodies do not affect interferon-mediated transcription, at least for the genes studied.

4.2.2.3 Locus-PML association in cell lines with different IFNγ responses

Finally, two renal cell carcinoma lines, CAKI-1 and CAKI-2 (HTB-46 and HTB-47), which have different responses to IFNγ, were studied (Dovhey *et al.*, 2000). The TAP/LMP genes have been found to be IFNγ -responsive in CAKI-1, but not in CAKI-2. This is due to a defect in the JAK tyrosine kinase protein in CAKI-2, resulting in a failure to phosporylate the STAT protein.

CAKI-1 and –2 cells were subjected to IFNγ treatment at 200U/ml for 24 hours. Expression of the 6 genes studied in the previous section was compared, using real-time RT-PCR, on IFNγ-treated and untreated populations of CAKI-1 and –2 cells. It was confirmed that the *LMP2*, *HLA-DRA* and *PSMB10* genes were upregulated in response to IFNγ in CAKI-1 but not in CAKI-2 (Figure 4.11). Similarly, the *COL1A1* and *PSMB7* genes are downregulated only in CAKI-1, although to a lesser magnitude.

ImmunoFISH experiments were then performed on the four different populations of cells (Figure 4.12, Appendix C.8). As CAKI-1 is a hyperpentoid cell line and CAKI-2 is hypotriploid, direct comparisons between the cell lines were not thought to be useful. Therefore, statistical analysis of locus-PML associations was confined to IFNγ-treated versus untreated cells. Again, correction for the PML-PML distances was performed. It was found that treatment with IFNγ did not affect the locus-PML association for the TAP/LMP locus or the chromosome 6 centromere (used as control) for either cell line.

These experiments support my previous finding that PML bodies do not appear to play a role in the interferon responsiveness of certain genes. Furthermore, the spatial association of these genes with PML bodies is not affected by interferon treatment.

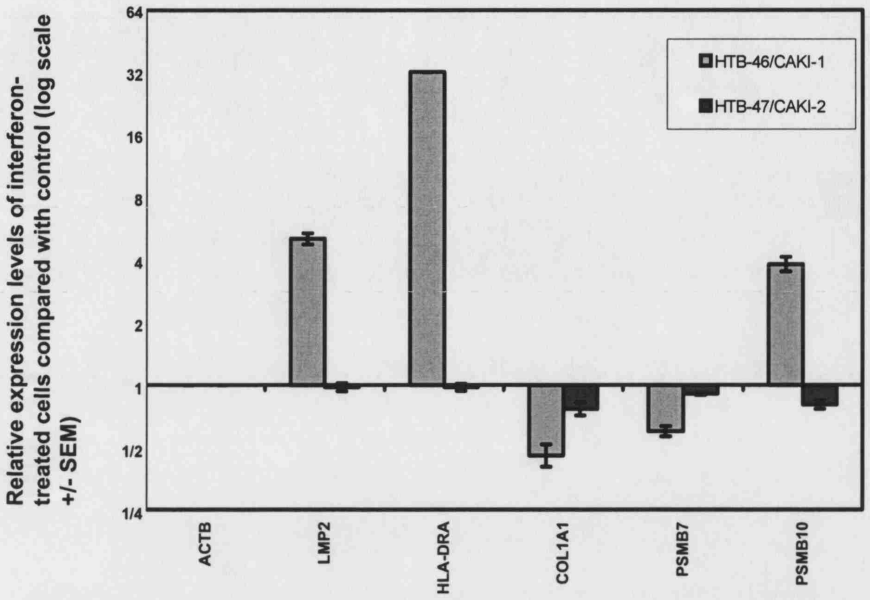

Figure 4.11. The effects of IFNγ on transcription of IFNγ responsive genes in CAKI-1 and CAKI-2 cell lines.

Graph showing the change in transcription levels of genes in cells treated with interferon, measured by real-time PCR, as compared with untreated cells. Five genes known to be IFNγ responsive were studied as well as the ACTB gene. The vertical axis represents the ratio of transcription level of a gene in interferon treated cells, to that of the control group (=1), on a $\log_2$ scale. The CAKI-1 cell line was interferon-responsive, as shown by the upregulation of the LMP2, HLA-DRA and PSMB10 genes, with downregulation of the COL1A1 and PSMB7 genes. This effect was not seen in the CAKI-2 cell line.

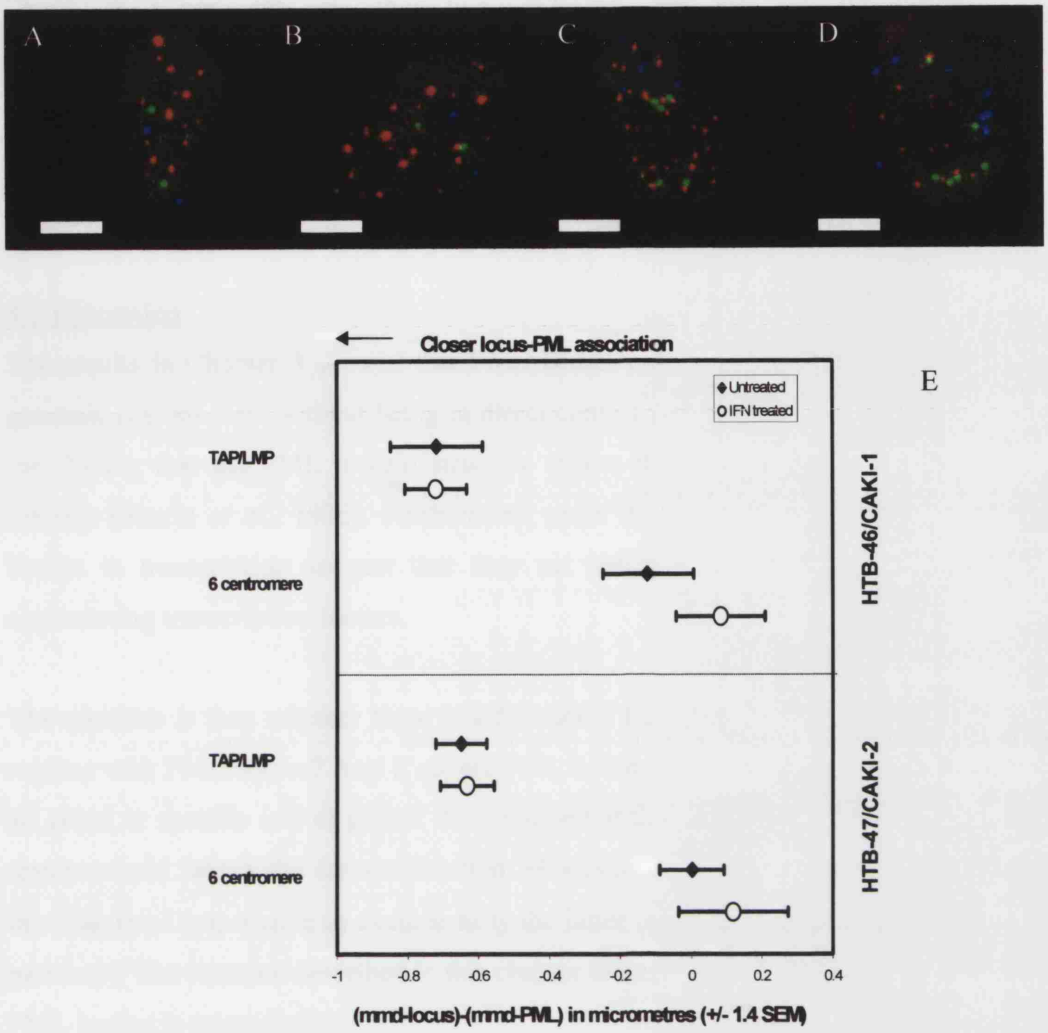

Figure 4.12. The association of PML bodies with genes in the CAKI-1 and CAKI-2 cell lines.

CAKI-1 (A,B) and CAKI-2 (C,D) cell nuclei showing the TAP/LMP locus (green), chromosome 6 centromere (blue), and PML bodies (red). The CAKI-1 cell line was hypotriploid, and the CAKI-2 cell line was hyperpentoid. B and D were from cells treated with IFNγ. The solid line represents 5 micrometres.

E: Locus-PML distances for different loci were compared in treated and untreated cells. The (mmd-locus) – (mmd-PML) on the X-axis represents the distance to which a locus is further or closer to the nearest PML body compared with the PML-PML distance, with error bars showing 1.4 standard errors. Unpaired T-tests were performed between the populations of treated and untreated cells for each cell line. Neither TAP/LMP nor the chromosome 6 centromere loci showed any statistical difference in association with PML bodies in either cell line.

Finally, the experiments using these two cell lines addresses the question raised above. That is, the PML bodies form in response to the general transcriptional activity of a region, rather than in response to acute changes in transcription. Furthermore, the presence of PML bodies in a region does not influence transcription in that region, as seen in CAKI-2.

4.3 Discussion

The results in Chapter 3 showed that PML bodies can spatially associate with certain genomic regions even without being in direct contact with them. This is consistent with the finding that the PML protein structure makes it unlikely for it to bind to DNA directly (Saurin *et al.*, 1996). Furthermore, most studies examining the role of PML bodies in transcription suggest that they act indirectly as depots by recruiting or sequestering transcription factors.

The question is then whether there is a functional basis for the association of specific regions with PML bodies? And if so, are PML bodies involved in basal transcription of all genes or specific sets of genes? As discussed at the end of Chapter 3, the statistical results would favour the former situation. However, as mentioned in the beginning of this chapter, I was unable to exclude fully the latter (especially with regard to the IFNγ pathway). The research described in this chapter therefore addressed the possible role of PML bodies in transcription.

4.3.1 PML bodies and uninduced (basal) transcription in general

The proposed role of PML bodies being involved in general transcription has been discussed in Chapter 1 and Section 4.1.1. To summarise, the presence of general transcription factors, such as RNA polymerase II and CBP, or transcription foci around PML bodies would suggest such a role (LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000). However, PML bodies cannot be crucial to the general transcriptional machinery, as evidenced by the viability of PML -/- cells and the differences in PML expression in certain cell types. However, this still does not mean that PML bodies do not participate in transcription. In particular, if PML bodies serve purely to degrade excess or inactive transcription factors, alteration of PML expression, at least in the physiological setting, may not lead to detectable changes in transcription levels.

In order to study the role of PML bodies in general transcription, semi-quantitative RT-PCR was performed for 49 genes, of the total of 54 loci studied on the autosomal chromosomes by immunoFISH. Expression level changes were measured following RNAi treatment. Because of cell population variations both within and between samples of cells harvested, as well as variations in the experimental procedure and intensity of bands captured, non-specific changes in mRNA levels were to be expected. This is seen in the large standard error of the mean for the experiments, when different cDNA samples were tested. To confirm the results, quantitative real-time RT-PCR was performed on a subset of 12 genes, for both RNAi and overexpression studies. Wide inter-sample variation still persisted, suggesting that cell population differences remain an important factor. In addition, several shortfalls have to be mentioned in these experiments:

1. The knockdown of PML bodies may not affect the transcription of a gene (even one which is closely associated with PML bodies), as its effect is on another nearby target gene. This hypothesis cannot be tested fully, unless the transcription levels of all genes in a statistically PML-associated genomic region were studied. However, the regression analysis in Chapter 3 suggests that PML bodies associate with an entire region, rather than with any specific gene within a region.
2. PML knockdown is not complete in the RNAi studies. I estimated that RNAi treatment resulted in up to 70% reduction of PML expression, but only 50% of cells showed complete knockdown. Using real-time PCR, changes in mRNA expression can only be resolved on a log2 scale, that is, only a two-fold increase or decrease of expression can be detected. This is therefore at the limit of resolution of the RNAi effects. However, RNAi studies consistently had no obvious effect on expression in different cDNA samples tested.
3. The experiments with overexpression of PML protein are prone to many potential artefacts, which will be discussed in more detail in Section 4.3.3. For example, foreign proteins that are known to accumulate in PML bodies (Mearini *et al.*, 2004; Tsukamoto *et al.*, 2000) may themselves induce the expression of PML, as well as trigger an upregulation of the MHC by affecting immunoproteasome activity. Hence, overexpression studies with PML must be interpreted with caution.

Due to these shortcomings, it was crucial to limit the interpretation of the results. For the purposes of this study, therefore, it was more important to determine simply if genes which were closer to PML bodies were more likely to be affected by PML protein (and PML body) knockdown or overexpression. No consistent effect of RNAi treatment was seen for genes which were more closely associated with PML bodies, compared with those less associated. Likewise, the findings from real-time RT-PCR experiments on NB4 cells did not show any variations consistent with the locus-PML association (albeit shown in a different cell line). There was upregulation of the *LMP2* gene in ATRA-treated NB4 cells, but this was not reflected in other genes which were PML body-associated, such as *ABC2*.

I have therefore shown that PML bodies are not directly involved in regulating transcription of genes with which they are closely associated. This does not exclude the possibility that PML bodies may be involved in transcription more indirectly, which will be discussed in Section 4.3.3.

4.3.2 PML bodies and the IFNγ pathway

In the research described in this chapter, I also explored the role of PML bodies in IFNγ -mediated transcription. Firstly, I found no increased association of PML bodies with several of the interferon-upregulated genes after IFNγ treatment of MRC5 cells. It must be noted that the regions studied already had high degrees of PML association. The statistical method used here takes into account the increase in the number of PML bodies in the nucleus, which is particularly important when assessing the effects of treatments to cells. Furthermore, negative control genes were used to exclude fully the changes in PML body numbers. The results were confirmed in the CAKI-2 renal carcinoma cell line, which showed that cells that were unresponsive to interferon retained the spatial association of PML bodies with the TAP/LMP locus.

These results appear to be in conflict with previous studies that suggested that interferon treatment led to an increased association of PML bodies with transcription foci or with DNA helicase II. For example, Kiesslich *et al* showed that IFNγ treatment of cells increased the association of PML bodies with transcription foci, labelled with fluorouridine, from a baseline level of 30% to 80% (Kiesslich *et al.*, 2002). Fuchsova *et al* found that IFNγ treatment led to the percentage of PML bodies incorporating DNA helicase II increasing from 47% to 81% (Fuchsova *et al.*, 2002). The assessments in

both studies were done based on direct contact between the foci (of transcription or DNA helicase II respectively) and PML bodies. Both studies varied from mine in several ways. Their main drawback was the lack of correction for the increase of PML bodies, which, as I have shown, can lead to artificially high association measurements. Furthermore, Kiesslich *et al* used IFNγ at a much higher dose of 2500U/ml, while Fuchsova *et al* used IFNα.

My immunoFISH data, however, do not in entirely discount a role for PML bodies in mediating IFNγ-responsiveness. One possibility is that interferon-induced genes may be regulated by interferon mediators sequestered and released by PML bodies. I attempted to perform immunofluorescence to determine whether PML bodies colocalise with STAT1α protein, IRF-1 or CIITA (results not shown). The results were inconclusive, because the STAT1α protein was found to be diffuse throughout the nucleus, while the IRF-1 and CIITA proteins were poorly visualised using the antibodies that were available.

RNAi studies were then performed to determine if these treatments affected the interferon response. Five genes were selected (*LMP2*, *PSMB7*, *-B10*, *HLA-DRA* and *COL1A1*) based on their IFNγ response, with the *ACTB* gene used as a control. The *LMP2* gene in the TAP/LMP cluster is particularly interesting with regard to the functional aspect, since this cluster encodes proteins which play a central role in the presentation of MHC class I molecules at the cell surface (van Endert, 1999). Expression of these genes was reported to be upregulated following transfection of PML protein into mouse tumour cells with defective antigen presentation (Zheng *et al.*, 1998).

In my experiments using a human cell line (MRC5), it was found in the last chapter that PML bodies are not themselves sites of transcription for the *LMP2* gene. Section 4.2.1.1 also showed that the uninduced transcription level of *LMP2* was not affected by RNAi treatment. I now find that PML knockdown by RNAi does not alter the response of LMP2 or the other IFNγ-responsive genes tested (*PSMB7*, *-B10*, *HLA-DRA* and *COL1A1*) to IFNγ treatment. This was further confirmed by FACS (fluorescence-activated cell sorter) experiments, which showed that MHC class I cell surface antigen induction by IFNγ was also not altered by RNAi treatment (results not shown). These

findings were consistent with a recent study where it was shown that PML is not directly involved in the regulation of MHC class I antigen expression at the cell surface (Bruno *et al.*, 2003).

Thus, the RNAi studies performed in this thesis indicate that despite previous inferred roles, PML bodies do not mediate the transcriptional response to IFNγ. Unfortunately, studies involving overexpression of PML could not be applied to IFNγ-responsiveness studies, due to the non-specific upregulation of PML protein and the MHC antigen presentation machinery by the transfection process itself. This does not necessarily discredit previous studies on *other* pathways using PML overexpression, although such studies have to be interpreted with caution (see next section).

4.3.3 Other roles of PML bodies in transcription

I have excluded, to a large extent, an active role of PML bodies in basal or uninduced transcription of genes with which they are associated, as well as in mediating interferon-induced transcription. Do PML bodies then have a role in regulating transcription of genes in other pathways, as indicated by numerous other studies (see Chapter 1)?

Regulation of transcription of specific genes by PML bodies has been previously reported to occur by the accumulation and release of transcriptional activators and repressors from PML bodies in a controlled manner. Previous research implicating PML bodies, directly or indirectly, in specific transcription include studies on Sp-1 (Vallian *et al.*, 1998a), NF-kB (Wu *et al.*, 2002a), nuclear hormone receptors (Baumann *et al.*, 2001; Wu *et al.*, 2002b), Daxx (Lehembre *et al.*, 2001), p53 (Guo *et al.*, 2000) and AP-1 (Vallian *et al.*, 1998b), as well as post-transcription such as eIF4E mediated mRNA transport (Cohen *et al.*, 2001). One of these possibilities that I have not explored, but which is consistent with the association of PML bodies with the MHC transcription, is that PML bodies affect MHC expression via NF-kB. The NF-kB transactivator is a mediator of TNFα, and was recently found to associate with PML protein (Wu *et al.*, 2003b), resulting in its repression. The promoter regions of the MHC class I and II genes have putative binding regions for NF-kB (Gobin *et al.*, 1998; Mansky *et al.*, 1994).

However, many of these studies, including those involving NF-kB, are not directly comparable to mine, since they use co-transfection of both exogenous PML protein and

various transcription factors to affect transcription levels. (In my experiments, transfection of PML was combined with IFNγ treatment of the cells, rather than the co-transfection of an IFNγ mediator, for example STAT1α.) As described above, there are several drawbacks of overexpression studies:

1. Non-specific changes in gene expression occur with transfection of DNA. Thus, overexpression studies in general may be prone to artefactual results. However, many of the studies described have used appropriate controls and showed that only PML-containing vectors have an effect on the expression of a gene.
2. In the studies involving Sp1 and AP1, associations of these transcription factors with the PML protein was shown only *in vitro*, by immunoprecipitation. This is not equivalent to studies on PML bodies *per se*.
3. The dosages of such transient transfections of PML are variable. A review of the literature showed only one paper in which cells were selected where the expression level of transfected PML matches that of endogenous expression (Cohen *et al.*, 2001). Thus, many such studies are likely to be unphysiological. The increased numbers of PML bodies in a nucleus may have non-specific effects that are again artefactual.
4. Related to point 3, the overexpression of different transcription factors to specifically activate a pathway is likewise unphysiological. These studies could only show that PML bodies/protein facilitate(s) or repress(es) a specific artificially induced transcription pathway, but do not determine if they are essential for the normal pathways or expression of the target genes. It may be that PML bodies only regulate transcription during specific overexpression or unusual activation of a particular pathway, rather than basal transcription or physiological activation.

If other specific pathways were to be excluded, what would be the role(s) of PML in transcription? One possibility is that PML bodies coordinate multiple pathways via a common downstream event, which would explain the presence of both specific and general transcription factors within them. For example, the transcriptional transactivator CBP, which also has histone acetylator activity, is found within PML bodies (Boisvert *et al.*, 2001; LaMorte *et al.*, 1998; von Mikecz *et al.*, 2000), where it appears to interact with the PML protein (Doucas *et al.*, 1999; Pearson *et al.*, 2000). CBP is suggested to mediate multiple pathways involving jun, CREB, E1A, E2F, NF-kB and nuclear hormone receptors (Chan and La Thangue, 2001; Vo and Goodman, 2001), which have

been variously linked to PML bodies. Alternatively, it may be that PML bodies adapt to a transcription pathway that is currently activated or dominant, as more of the general transcription factors are recruited into that pathway. This may also explain the fact that treatment with IFNγ caused the proportion of PML bodies containing DNA helicase II (involved in the interferon pathway) to increase (Fuchsova *et al.*, 2002), as more PML bodies become involved in the interferon response.

In addition to CBP, other chromosome interaction proteins have been found associated with PML bodies, including HP1, and HMG1 and 2 (see Chapter 1). PML bodies may act to affect chromatin structure to promote transcription responsiveness, rather than directly activating transcription. This was suggested by Spector and colleagues, who observed that PML bodies associate with loci where foreign genes were inserted, but only when the genes became bound by specific enhancer/promoter proteins (Tsukamoto *et al.*, 2000).

I have also not explored the possibility that PML bodies are involved in post-transcriptional events, as proposed by others (Borden, 2002). Several studies have shown that nascent RNA can occur either around or within PML bodies (Boisvert *et al.*, 2000; LaMorte *et al.*, 1998). In the RNA-FISH experiments described in Chapter 3, there appears to be more contact between PML bodies and mRNA transcripts than with the genes themselves. This, together with the finding that the eukaryotic initiation factor 4 (EIF4) involved in RNA transport, is found in PML bodies (Cohen *et al.*, 2001; Strudwick and Borden, 2002) indicates that control of gene expression by PML may also occur downstream of the transcription process by regulating RNA metabolism. Other post-transcriptional events include RNA splicing, degradation of excess copies, or preparation for transport of mRNA. However, as described above, MHC class I protein expression at the cell surface appears to be unaffected by the presence of PML bodies (Bruno *et al.*, 2003).

4.3.4 A possible basis for the PML-genome association

Notwithstanding the possible other roles of PML bodies in transcription, the RT-PCR results presented here do not support a regulatory role of PML bodies in either general or interferon-mediated transcription. What then can we infer from the immunoFISH data in this chapter? The conclusion from the immunoFISH analysis of IFNγ treatment of the MRC5 and CAKI-1 cells was that PML bodies associate with the MHC

(TAP/LMP) region, regardless of the acute changes of transcription status in this region. While this may be contradictory to the effect seen with the histone cluster in S phase cells (Section 3.2.3.5), two features distinguish the histone cluster. Firstly, the S phase presents a dynamic period for PML bodies (Koken *et al.*, 1995); and secondly, the histone cluster forms a continuous region of coordinated transcription.

It was also observed that the newly formed PML bodies show a preference for the MHC locus in NB4 cells following ATRA treatment. Although the *LMP2* gene was also seen following ATRA treatment, the RT-PCR data from the RNAi and overexpression studies in this chapter suggest that the association of PML bodies with the MHC in NB4 cells forms in *response* to the transcriptional activity in that region. It is further suggested, based on the previous paragraph, that this response is to the general transcription in that region, rather than the ATRA-induced transcription changes. This is also seen in the CAKI-2 cells, in which PML bodies associate with the MHC region, despite being unresponsive to IFNγ.

Therefore, it is proposed that PML bodies associate with a region in response to the pre-existing transcriptional state, being more likely to be found in a transcriptionally permissive or 'fertile' region.

To summarise, although PML bodies have been shown to associate with regions of high transcriptional activity, including several subsets of functionally related genes, they do not appear to be important in affecting transcription in these regions. This would suggest the PML bodies form in response to transcription, rather than influencing it. It might also be that PML bodies are involved in degrading or sequestering transcription factors or other proteins in the vicinity of active transcription, but such actions do not have a regulatory role under physiological conditions. Rather, they may simply serve to remove excess proteins, endogenous or foreign.

Chapter 5: Nuclear organisation of PML bodies in S phase

5.1 Introduction

While performing the experiments described in Chapter 3, I noted that during S phase PML bodies often associate with replication foci. This prompted me to examine the relationship between PML bodies and replication foci in more detail.

5.1.1 PML bodies and replication

In contrast to other proposed functions of PML bodies, their possible role in DNA replication remains little studied. There is, however, evidence that they do have such a role. It has long been recognised that PML bodies are distributed in a characteristic pattern depending on the cell type and the stage of the cell cycle (see Chapter 1)(Koken *et al.*, 1995). In S phase, PML bodies become more speckled and numerous. During infection of certain viruses such as HSV, viral DNA replication is thought to occur in and around PML bodies (see Table 1.4). Several proteins involved in cell cycle control, including pRB and p53 (see Table 1.3), have been found to accumulate in PML bodies, suggesting that these bodies may also coordinate entry into S phase. Finally, cells overexpressing PML have been noted to have a delayed entry into S phase, while PML knockout cells have an increased proportion of cells in S phase (see Section 1.3.7.2) (Mu *et al.*, 1997; Wang *et al.*, 1998a).

Grande *et al* showed that PML bodies have a high association with replication foci during mid- to late S phases (50-80% of PML bodies in contact with foci), but not in early S phase (Grande *et al.*, 1996). Furthermore, PML bodies were found to be adjacent to, rather than colocalising or overlapping with replicating DNA. The authors suggested that PML bodies were either involved in DNA replication only in middle-late S phase, or else are associated with chromatin which is replicated specifically at that period. However, a drawback of this study was that analysis was only carried out in one bladder cancer cell line. As will be discussed later, the pattern of replication foci varies depending on the cell line used. In another study by the same group, novel nuclear structures containing parathymosin were noted to associate with replication foci in early S phase (Vareli *et al.*, 2000). As these structures did not colocalise with PML bodies, it was presumed that PML bodies were not involved in early DNA replication.

To study further the role of PML bodies in DNA replication, it is first important to understand the regulatory processes which occur in replication, both temporally and spatially.

5.1.2 Replication Origins

As mentioned in Chapter 1, DNA replication during S phase occurs at multiple specific sites, or origins, in eukaryotes. The number of origins varies from 300-400 in S cerevisiae, compared with an estimated 10 000 in humans (Gilbert, 2001; Wyrick *et al.*, 2001). The temporally organised 'firing' of clusters of origins gives rise to early and late replication banding patterns which are visualised on metaphase chromosomes. These bands correspond to differences in the genomic regions, suggesting that timing and positions of replication origins are functionally important with regard to gene activity.

The earliest studies to characterise replication origins were performed in S cerevisiae, where they were termed autonomously replicating sequences (ARSs) and found to consist of AT-rich sequence blocks of 100-200bp (Marahrens and Stillman, 1992). When inserted into plasmids, these sequences functioned as origins of replication allowing the plasmids to replicate (Brewer and Fangman, 1987). In contrast, replication origins of higher eukaryotes have been more difficult to identify (reviewed in Biamonti *et al.*, 2003). Putative origins have been identified in numerous genes, including the human β-globin (Aladjem *et al.*, 1998) and the CHO dihydrofolate reductase (DHFR) genes (Dijkwel *et al.*, 1994). However, while some origins are mapped to a single site within 2kb (Toledo *et al.*, 1998), others are present as multiple origins within an initiation zone, such as the 28kb region between *DHFR* and *2BE2121* genes (Kobayashi *et al.*, 1998). This suggests that in higher eukaryotes, sequence alone is not enough to specify where replication should begin. Instead, chromosomes contain many motifs that can act as origins although some are preferred. Multiple motifs are proposed that contribute to origin function, including Alu elements, DNA binding elements, SARs, ARS-like elements, Z DNA, and pyrimidine tracts (reviewed by DePamphilis, 1998; DePamphilis, 1999). Epigenetic factors may also determine the probability of an origin site being used, as well as the timing of initiation at a site (see below).

Although the sequences that contribute to replication origins vary between species, the protein complex that interacts with these origins, the origin recognition complex (ORC)

remains evolutionarily conserved (Gavin *et al.*, 1995). Well-conserved cell cycle dependent events occur at the ORC resulting in DNA replication (reviewed in Diffley and Labib, 2002; Kelly and Brown, 2000). The ORC complex (consisting of subunits 1-6) remains at origin sites through mitosis. Then, early in G1, the ORC complex recruits cdc6, cdt1, and the MCM (2-7) group of proteins to form a pre-replicative complex (pre-RC), followed by cdc45 and Sld3 to form the pre-initiation complex (pre-IC). During S phase, the expression and modification of various cyclins and cyclin-dependent kinases (in metazoans, this is the cyclinA/E-cdk2 complex) results in conformation changes in the pre-IC, with recruitment of the various replication factors, including DNA polymerases, to trigger replication.

5.1.3 Replication origin usage and timing

During development, the number of origins used decreases as cellular differentiation progresses. In early *Xenopus* embryos, S-phase takes 15 minutes (in a 30 min cell cycle). In these embryos, it was found that multiple origins within the non-transcribed rDNA genes spaced at 9-12kb apart were fired synchronously (Hyrien and Mechali, 1993). Following the mid-blastula stage, when transcription of the rDNA genes begins, the replicon size increases (as does the length of S-phase) and becomes more variable (Hyrien *et al.*, 1995). The mechanism of this reduction in origin usage in development is not known. However, it has been suggested that chromatin conformation and/or epigenetic factors play a role (reviewed by Coffman and Studzinski, 1999; DePamphilis, 1998). In addition, the nuclear environment may play a part. Anglana *et al* mapped a region downstream of the *GNA13* gene in Chinese hamster cells, and found a single high frequency 'firing' (primary) origin, as well as several low frequency (secondary) origins (Anglana *et al.*, 2003). The frequency with which the secondary origins were activated was found to depend on the nucleotide pool.

It is also recognised that different origins 'fire' at different times, as shown by the early and late replication of euchromatin and heterochromatin respectively. This also indicates that transcriptional status and replication time are linked, as seen by the earlier replication of active genes than inactive genes (Hatton *et al.*, 1988; Holmquist, 1987). Furthermore, it has been found that house-keeping genes often replicate in the first half of S-phase in many cell types (Hatton *et al.*, 1988). In contrast, tissue-specific loci can be divided into two groups. The first group replicates early in S-phase whether they are expressed or not (Azuara *et al.*, 2003; Hatton *et al.*, 1988). The second has a

developmentally regulated pattern of replication whereby they replicate early in expressing cells, and late in non-expressing cells. An example of this is the human β-globin locus, where over 200Kb of DNA is early replicating in expressing erytholeukemia cells but late replicating in lymphocytes and HeLa cells (Dhar *et al.*, 1989).

The precise role of transcriptional activity in replication timing remains elusive (Gilbert, 2002). Fisher and colleagues have shown that insertion of transgenes into heterochromatin resulted in their late replication, but this was reversed following variegated transgene expression (Azuara *et al.*, 2003). Furthermore, the replication timing of the β-globin gene inserted into a late replicating region in the mouse genome was dependent on its orientation, with early replication resulting from a transcription-permissive orientation (Lin *et al.*, 2003a). This did not require the presence of either the promoter of the gene or a replication origin in the insert. Epigenetic states may also affect replication timing. For example, histone acetylation and DNA methylation may be involved in altering the replication timing of certain genes, including those on the inactive X chromosome (Hansen *et al.*, 1996; Vogelauer *et al.*, 2002), although a study on imprinted genes showed that DNA methylation was not responsible for the asynchronous replication firing of these regions (Gribnau *et al.*, 2003). Binding sites of transcription factors are often located proximal to replication origins in yeast and viruses, and the presence of these factors facilitates replication initiation (reviewed by Coffman and Studzinski, 1999).

5.1.4 Replication patterns in S phase

The patterns of DNA replication in the nucleus follow a distinct sequence over the course of S phase. In 1992, O'Keefe *et al.* used a combination of fluorescence, confocal and electron microscopy, primarily in CHO cells, to define five distinct patterns of DNA replication based on the nuclear distribution of incorporated BrdU (O'Keefe *et al.*, 1992). Cells were pulse-labelled with BrdU, and subsequently detected in fixed nuclei using anti-BrdU antibodies. The labelling of nascent DNA has been shown to colocalise with replication foci, as labelled with PCNA (Sadoni *et al.*, 2004). Therefore, the distribution of the nascent DNA containing BrdU thus corresponds to the pattern of replication foci. In early S phase, replication starts at several hundred small foci distributed throughout the nucleoplasm, excluding the nucleolus. As S phase continues, replication of euchromatin decreases and replication of heterochromatin begins. The

foci decrease in number but increase in size, and become localised near the nuclear envelope and around the nucleolus. At the end of S phase, replication is confined to a few very large replication foci in domains deep within the nuclear interior. Dual colour labelling of replication foci suggests that replication persists at a particular focus for about an hour (Manders *et al.*, 1992).

Similar patterns are seen in a variety of cell types, including foetal fibroblasts, B-cells and plant cells (Nakayasu and Berezney, 1989; Neri *et al.*, 1992; Sparvoli *et al.*, 1994). Such conservation across different cell types and completely unrelated organisms suggests that this distribution is a basic feature of the temporal and spatial organisation of DNA replication, and indeed of interphase chromatin in higher eukaryotes (Zink *et al.*, 1999; Zink *et al.*, 1998). However, it was found recently that primary fibroblast cell lines (including MRC5, WI-38, IMR-90 and MEFs) show only a few punctate foci in early S phase (Kennedy *et al.*, 2000), before reaching a diffuse microspeckled pattern. In contrast, the microspeckled pattern is seen from the onset of S phase in cancer or immortalised cell lines (Kennedy *et al.*, 2000; O'Keefe *et al.*, 1992). This difference was suggested to be due the loss of order of replication timing in malignancy. Alternatively, the different observed patterns may be due to fixation methods, or different timings of S-phase periods as defined by the studies.

The discussion above has described the relationship between transcriptional status and replication timing. However, how is transcription spatially coordinated with replication during S phase itself? This is especially important in early S phase, where transcriptionally active genes are being replicated. Jackson *et al* used dual colour labelling to visualise transcription and replication foci simultaneously (Jackson *et al.*, 1993). This group found that replication and transcription foci tended to colocalise, even in the heterochromatic regions in late S phase (Hassan *et al.*, 1994). Berezney and colleagues however found using 3 dimensional imaging that the replication and transcription foci were distinct and separated from each other, forming distinct zones or networks (Wei *et al.*, 1998). It was therefore proposed that regions of transcription were 'switched off' to enable a wave of replication to pass by (Berezney, 2002). It was further proposed that such zoning occurs using the nuclear matrix (Wei *et al.*, 1999).

In the work described in Chapter 3, I found that for several regions, there was no statistical difference between G0/1 phases and S phases in the association of PML

bodies with specific loci, with the exception of the histone gene cluster in chromosome band 6p22, which contains genes largely transcribed in S phase. I have now extended this study specifically to the different stages of S phase, in order to determine whether there is a spatial organisation of PML bodies relative to the replication timing of these regions.

5.2 Results

5.2.1 Characterising the patterns of replication foci during S phase

To determine how the pattern of replication foci alters during S phase in MRC5 cells, unsynchronised MRC5 cells were pulsed with BrdU for 1 hour prior to fixation. The replication foci, identified by nascent DNA labelled with BrdU, were then detected by anti-BrdU antibodies and fluorochrome-tagged secondary antibodies. Four main patterns of replication foci were seen (see Figure 5.1,A-D, showing simultaneous labelling of PML bodies).

Next, to determine the order of the different patterns as cells progressed through S phase, dual labelling of replication foci over different time intervals was performed on unsynchronised MRC5 cells. For this, it was assumed that the pattern of replication (visualised as nascent DNA labelled with one fluorochrome) at a specific period of S phase persisted following washout and subsequent labelling with a second fluorochrome. Cells were first incubated with BrdU, which was then washed out, and after 2-6 hours, they were labelled by the scratch method using FITC- or TR-dUTP. Alternatively, cells were first scratch-labelled with fluorochromes-dUTP, washed and pulsed with BrdU. The BrdU antigen was detected using appropriate antibodies giving an alternative fluorochrome colour to the dUTP (See Figure 5.2.A-D for examples). Using this method, it was determined that each of the four patterns of replication foci occupied approximately 2 hours of S phase, although a gradual transition was observed between patterns. It must be noted however, that unlike BrdU labelling by incubation, scratch labelling only labels some of the cells in S phase. Therefore only cells labelled with both nucleotides were analysed.

The strategy of sequential labelling was also used to determine if patterns seen were specific to early or late S phase. For example, to show that a pattern was specific to early S phase, cells were labelled first with BrdU, followed by FITC -dUTP. Assuming

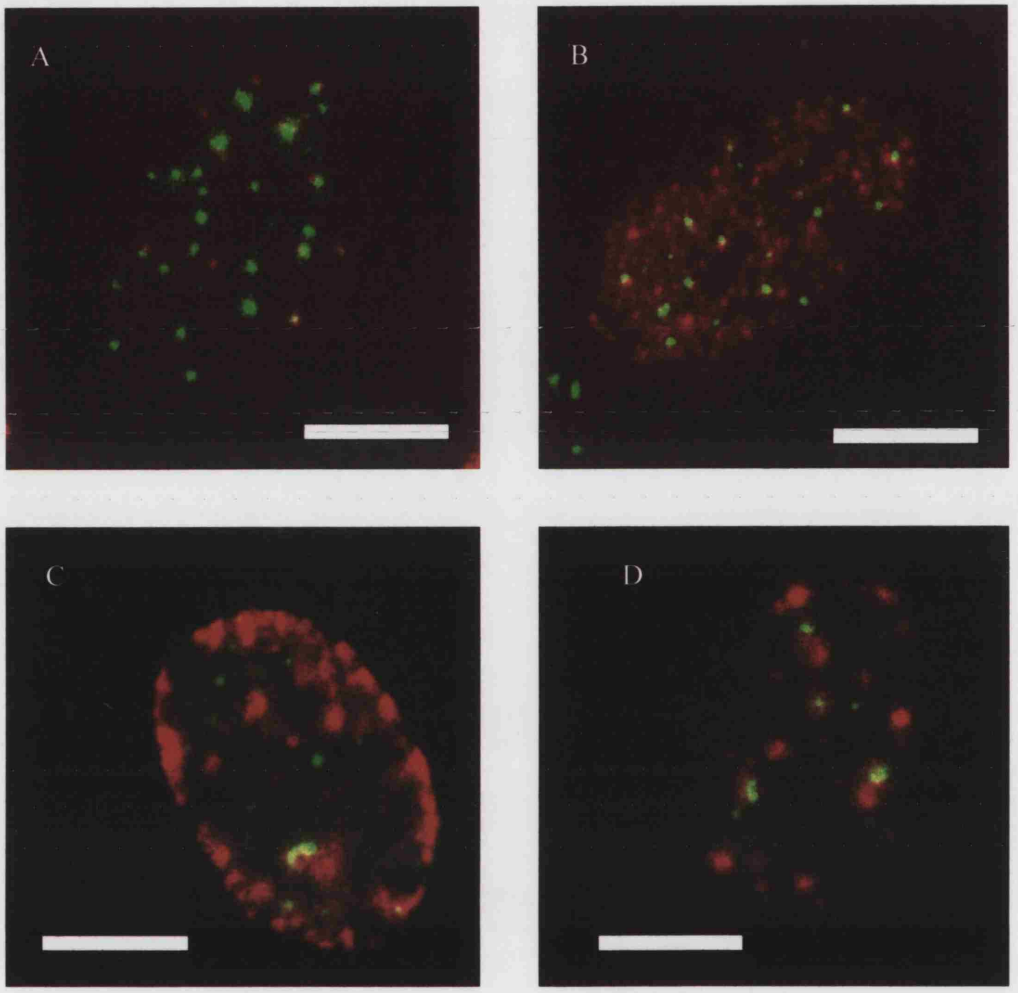

Figure 5.1. Patterns of replication foci during different stages of S phase.
A-D: MRC5 cells showing replication foci were labelled with BrdU, and detected with TR- labelled antibodies. PML bodies are in green. The solid line represents 5 micrometres.
A: Stacked image showing replication pattern S1, characterised by few small foci.
B: Stacked image showing replication pattern S2, characterised by diffuse replication foci throughout the nucleoplasm.
C: Single section image showing replication pattern S3, characterised by foci in the nuclear periphery and in the interior perinucleolar regions.
D: Single section image showing replication pattern S4, characterised by fewer larger replication domains.

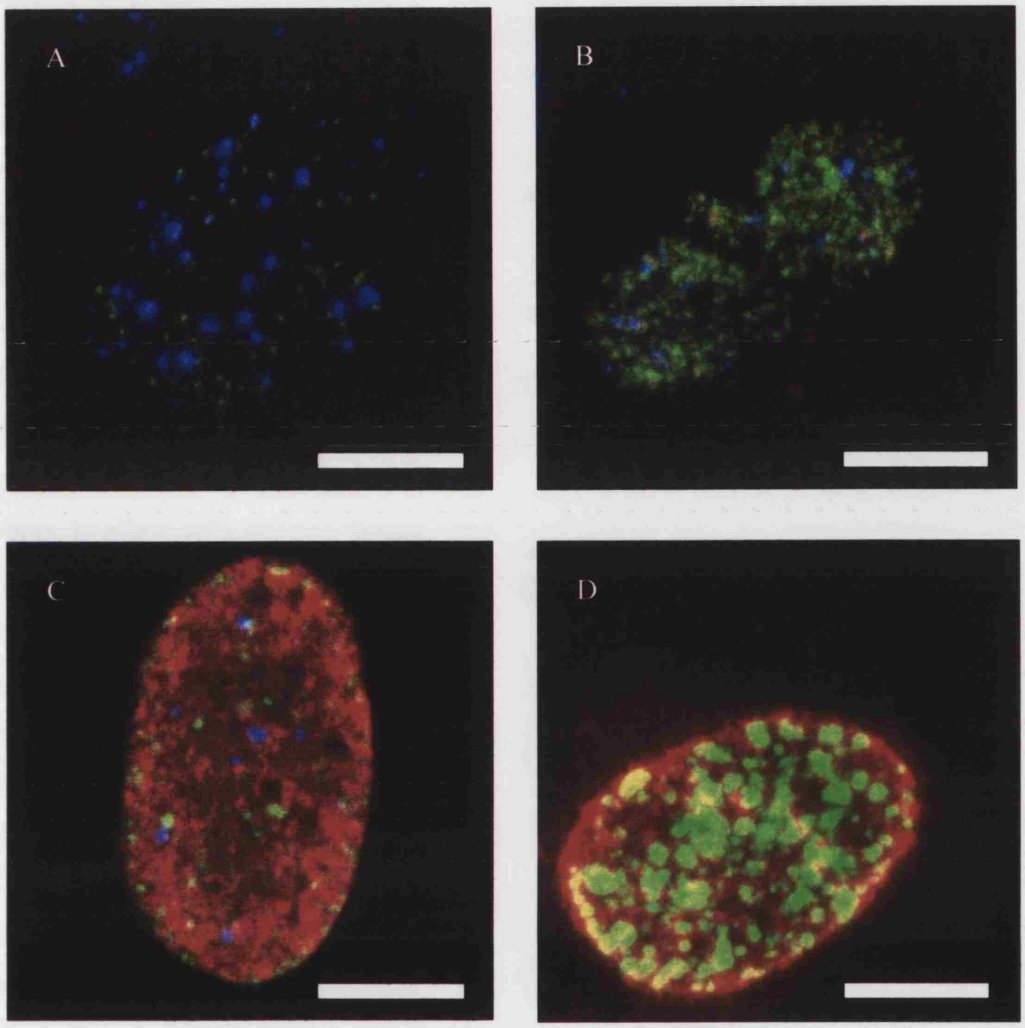

Figure 5.2 Timing of replication foci patterns determined by dual labelling.
A-D: MRC5 cells with replication pattern labelled with dual colour labelling, where cells were labelled first with BrdU (subsequently detected red with TR-labelled antibodies), washed out for 2 hours, and labelled green with FITC-dUTP. PML bodies are in blue. The solid line represents 5 micrometres.
A: Stacked image showing cell which was previously in G1 (showing no incorporation of BrdU), and entering S phase with a pattern of S1.
B: Stacked image showing cell showing a replication pattern of S1 followed by a pattern of S2.
C: Single section image showing a replication pattern of S2 followed by a pattern of S3.
D: Single section image showing a replication pattern of S3 followed by a pattern of S4.

that BrdU (subsequently labelled with Texas Red) will be incorporated in all S phase cells, MRC5 cells which showed only FITC fluorescence were considered to have been in G1 during the first period of labelling, and to have progressed into S phase during FITC-dUTP labelling. The reverse labelling (that is, cells were first labelled with fluorochrome-dUTP, followed by BrdU) was performed to detect and observe cells in late S phase.

To confirm whether the patterns observed were specific to the different periods of S phase, MRC5 cells were also synchronised using a double thymidine block. Cells were treated with thymidine for 18 hours to arrest them at the G1/S boundary, released for 8 hours, to allow cells arrested during mid-S to progress, and blocked by thymidine again for a further 18 hours. Different populations of cells were then released for 1-7 hours, and labelled with BrdU for the last hour of release before fixation and detection with appropriate antibodies. The results obtained broadly corresponded to those determined by dual labelling (see Figure 5.3.A-D). To ensure that the thymidine block at S phase was complete, blocked cells were checked prior to being released to see that they showed no BrdU incorporation.

The replication patterns obtained were thus classified into 4 main categories, S1-S4, in order of their appearance in S phase. The patterns observed are an amalgamation of the two series of patterns previously obtained by O'Keefe *et al* and Kennedy *et al* (Kennedy *et al.*, 2000; O'Keefe *et al.*, 1992) (Figure 5.1.A-D). In S1 phase, there are a variable number of discrete foci. In S2, the replication foci become diffuse within the nuclear volume. S3 is characterised by foci in the nuclear periphery and around the nucleolus, while S4 has a few large foci. These patterns were also seen in the AHB lymphoblastoid cells (Figure 5.4.A-D) and in HeLa cells (data not shown).

5.2.2 PML bodies associate with replication foci throughout S phase

I then examined the association of PML bodies with replication foci in MRC5 cells. Unsynchronised cells were labelled with BrdU immediately prior to fixation, and anti-BrdU and anti-PML bodies used for detection. Cells in phases S1 – S4 were identified from the patterns of their replication foci. For the phases S1, S3 and S4, the total number of PML bodies per cell and the number in direct contact with a replication focus were counted. This was performed by two independent observers and the average scores were used. Cells in S2 showed a diffuse pattern of BrdU incorporation, and thus, the

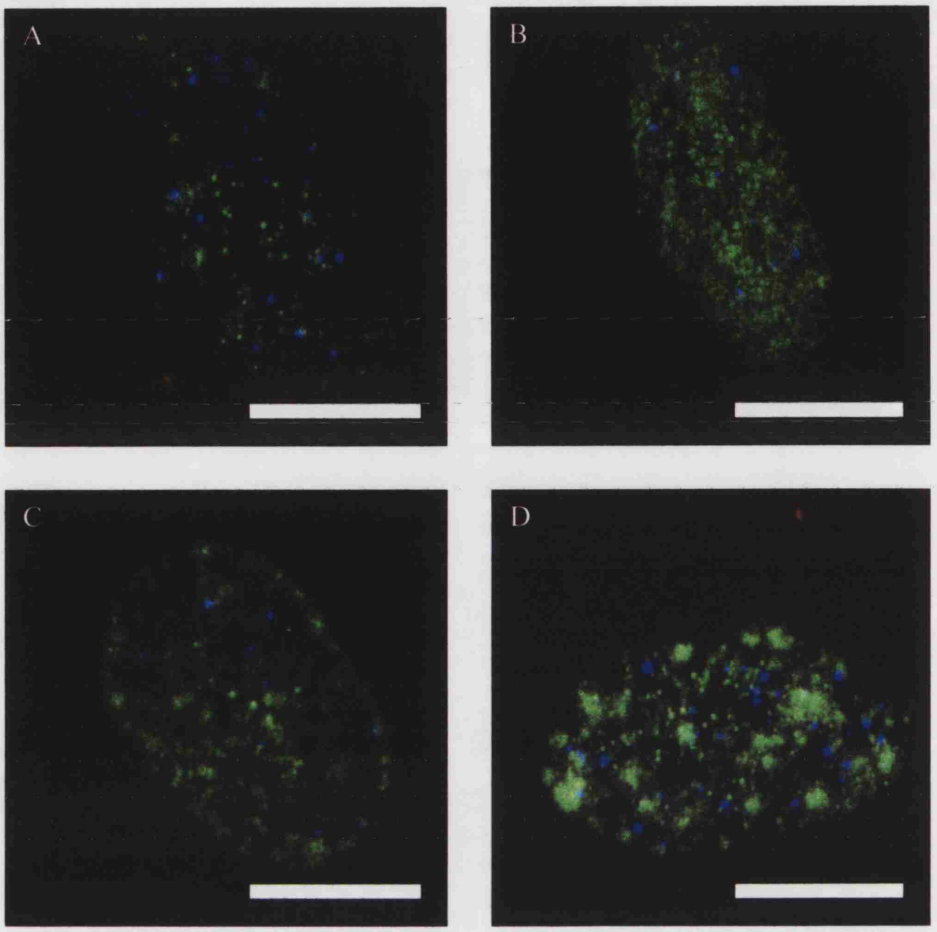

Figure 5.3. Timing of replication foci patterns determined by cell synchronization.
A-D: MRC5 cells where synchronized using double thymidine blocks, and released for a variable period of time prior to BrdU pulsing. Replication foci are shown in green, and PML bodies are shown in blue. The solid line represents 5 micrometres.
A: Typical pattern for cell which was released from thymidine block for 1 hour prior to BrdU pulse, showing the S1 pattern (stacked image).
B: Typical pattern for cell which was released for 3 hours prior to BrdU pulse (S2) (stacked image).
C: Typical pattern for cell which was released for 5 hours prior to BrdU pulse (S3) (single section).
D: Typical pattern for cell which was released for 7 hours prior to BrdU pulse (S4) (stacked image).

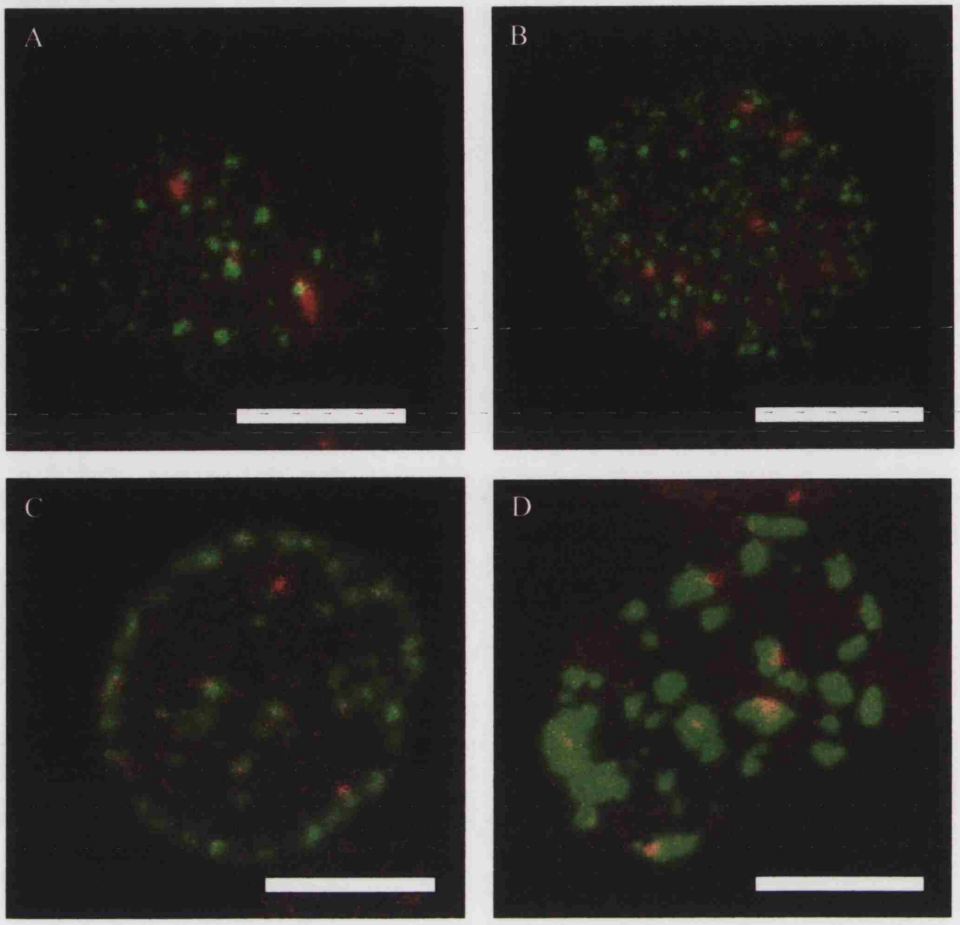

Figure 5.4. Pattern of replication foci during different stages of S phase in AHB cells.

A-D: AHB cells showing replication foci (BrdU) in green, and PML bodies in red. PML bodies were often associated with replication foci throughout S phase. The solid line represents 5 micrometres.

A: Replication pattern S1, characterised by few small foci (stacked image).

B: Replication pattern S2, characterised by diffuse replication foci throughout the nucleoplasm (stacked image).

C: Replication pattern S3, characterised by foci in the nuclear periphery and in the interior perinucleolar regions (single section).

D: Replication pattern S4, characterised by fewer larger replication domains (stacked image).

percentage of PML bodies associating with replication foci would be high due to the abundance of labelled nascent DNA. Therefore, cells in S2 were excluded from this scoring.

I found that a high proportion of PML bodies (60-70%) associated with replication foci in both early (S1) and mid-late to late (S3 and S4) phases (Table 5.1, see scores for washout periods of 0 hours). Even in S2, where the replication foci were more diffuse, PML bodies were often found in the vicinity of the strongest BrdU signals (see Figure 5.1.B). Furthermore, in some cells, the morphology of PML bodies was altered, and in extreme cases, a PML body formed a cap around a replication focus (Figure 5.1.C). Strikingly, in mid-late S3 phase, when replication foci were predominant in the nuclear periphery, with a few large foci in the interior, PML bodies were distributed likewise around the periphery with only a few in the interior (in association with the replication foci) (Figure 5.5). This pattern of association was also seen for the AHB lymphoblastoid cell line, although not quantified (Figure 5.4, A-D).

Thus, the proportion of PML bodies which associate with DNA replication sites in the later S phases was consistent with that previously published (Grande *et al.*, 1996). In the previous report, the association was seen in the middle-late S phase period, which corresponded to S4 in my study. However, unlike the observations in the previous report, I also found a strong association earlier in S phase.

5.2.3 The association of PML bodies with replication foci is dynamic

While the observed association of PML bodies with replication foci suggested a functional interaction, there were at least two alternative explanations: (a) that this apparent association is due to the confined volume of a nucleus, and (b) that PML bodies associate with certain replication foci due to particular properties in these genomic regions (e.g. early replicating regions which are more highly transcribed). In either situation, (a) or (b), the association of PML bodies with the pattern of replication foci will be static. That is, PML bodies would be seen to associate with these regions even after they were no longer replicating.

To exclude these possibilities, I performed experiments in which the BrdU was washed out after different time intervals prior to fixation (Figure 5.6.A-D). Cells that were pulsed with BrdU and immediately fixed showed foci that were actively replicating at

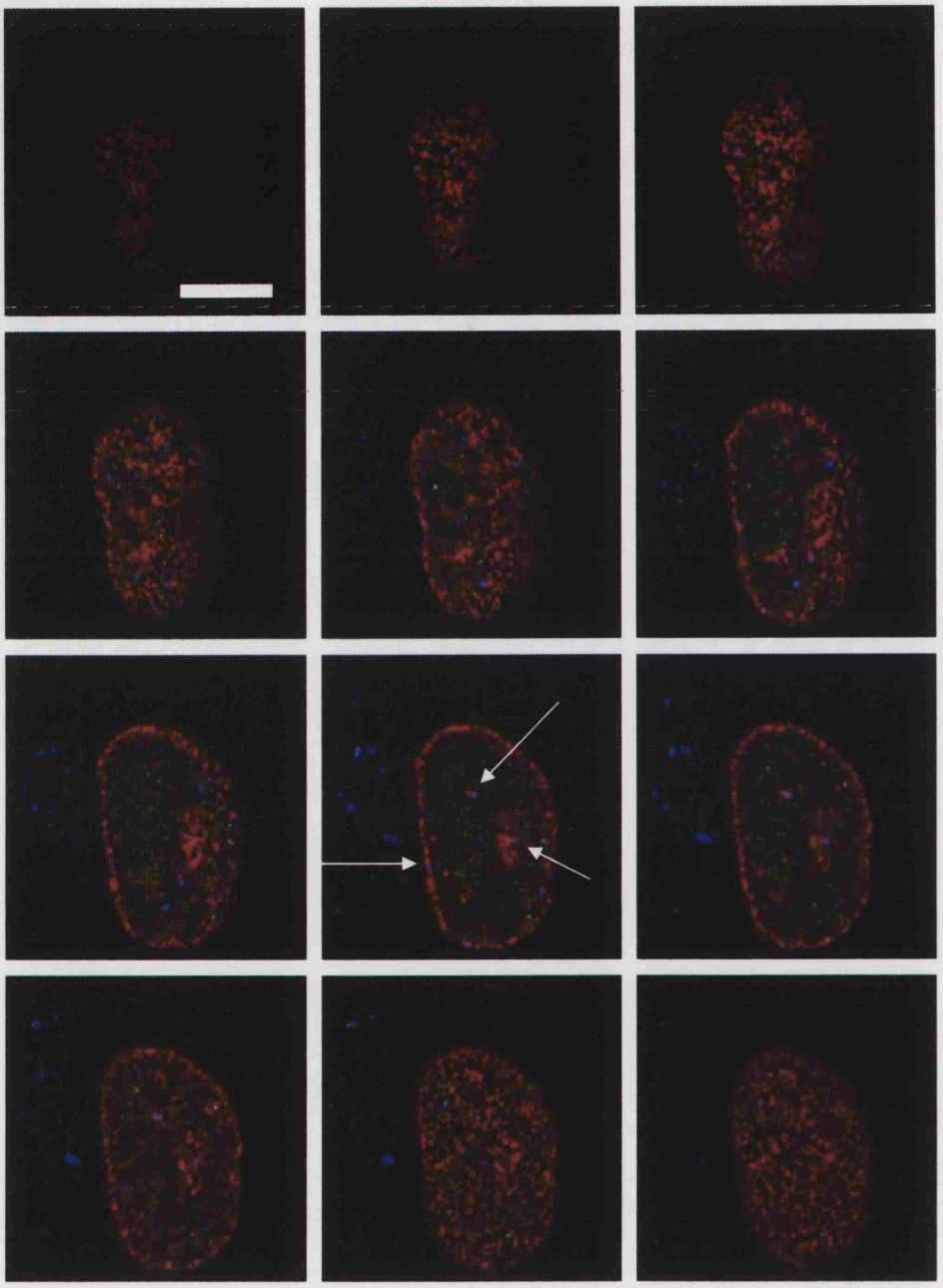

Figure 5.5. The association of PML bodies with replication foci visualized in 3D. Confocal sections through an MRC5 cell in S3 (mid-late S phase) at 0.4μm intervals. PML bodies were detected in blue, replication foci in red and transcription foci in green. Note that PML bodies are associated with replication foci in the nuclear periphery and with large replication foci in the nuclear interior (arrowed). The solid line represents 5 micrometres.

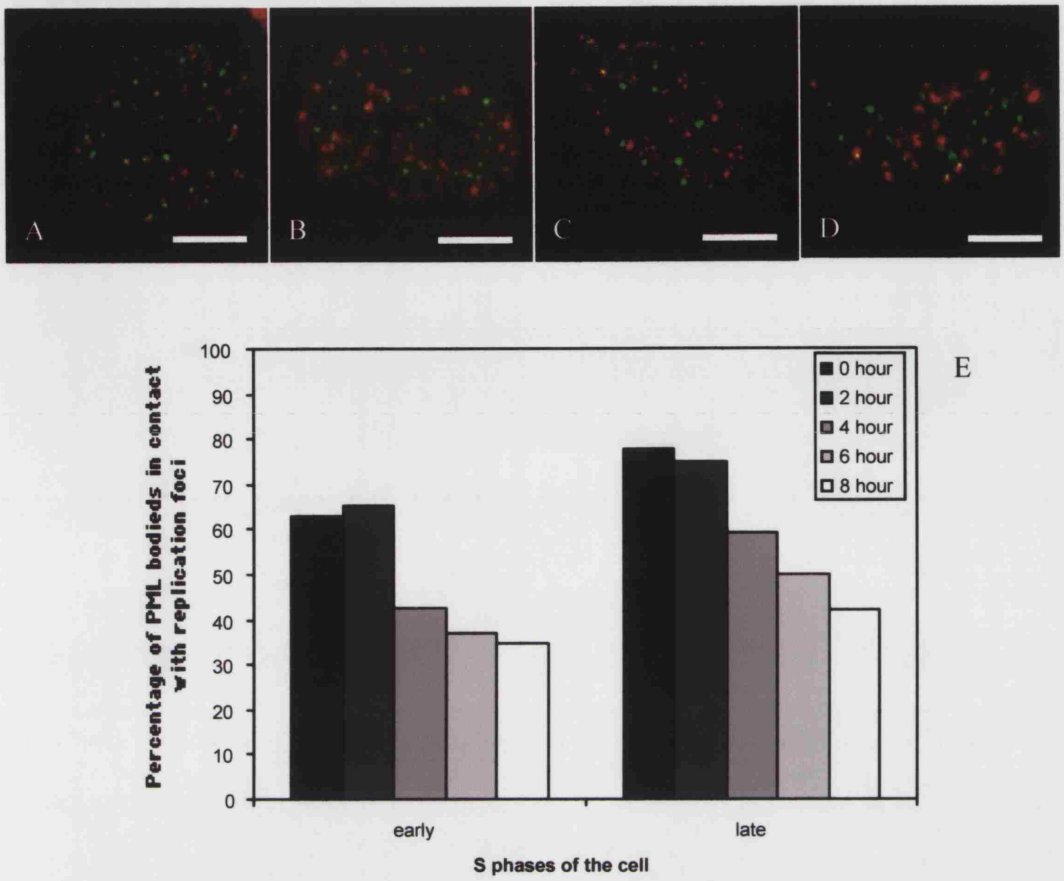

Figure 5.6. The association of PML bodies with nascent DNA after different washout times.

A-D: MRC5 cells were pulsed with BrdU for 1 hour to label nascent DNA at that time, followed by washout periods of up to 8 hours prior to fixation and labelling for PML bodies (green) and BrdU incorporation (red). Stacked images showing whole cell are shown. The solid line represents 5 micrometres.

A,B: Cells were fixed immediately after BrdU labelling. PML bodies associate partially with active replication foci, during both S1 (A) and S4 (B) periods of S phase.

C,D: Cells were labelled with BrdU 8 hours prior to fixation. PML bodies show less association with nascent DNA that were replicating foci 8 hours prior to fixation. In both S1 (C) and S4 (D) periods, fewer PML bodies were associated with the nascent DNA signals.

E: Graph showing the decreasing association of PML bodies with nascent DNA signals after different washout times. The proportion of PML bodies, which were in contact with labelled nascent DNA was visually determined. Populations of cells were also sub-divided into either early S phase (S1) or late (S3 and S4). S2 cells were excluded from this study as they had diffuse pattern of replication foci.

Table 5.1. The number and proportion of PML bodies in contact with replication foci at different stages of S-phase, and after different washout periods.

Washout period	Number of cells (n)	PML bodies (n)	PML/cell	PML/cell in contact	PML%
Early S (S1)					
0h	51	974	19.1	12	62.9
2h	38	707	18.6	12	65.2
4h	32	628	19.6	8.5	42.5
6h	32	579	18.1	6.3	37.1
8h	31	555	17.9	6.1	34.6
Late S (S3,4)					
0h	35	528	15.1	12	77.8
2h	27	685	25.4	19	75
4h	29	629	21.7	13	59.4
6h	33	619	18.8	8.9	49.9
8h	28	507	18.1	8.2	42.2

the time of fixation. In contrast, cells that had nascent DNA labelled with BrdU, following which the BrdU was washed out for variable periods of time, enabled replication foci from a period prior to fixation to be visualised. This technique was previously used by Tashiro *et al*, and the foci were termed "post-replicative chromatin" (Tashiro *et al.*, 2000). The nascent DNA sites thus labelled are no longer actively replicating at the time of fixation, but retain their characteristic replication pattern from that prior timeframe (as shown by dual labelling experiments, see Section 5.2.1).

I therefore wished to see if the pattern of PML bodies was dynamic in S phase, and also if there was a lag in the change in distribution (Figure 5.6.E, Table 5.1). I found that the association of PML bodies with replication foci tended to persist for 2 hours after washout, but the proportion of PML bodies in contact with labelled nascent DNA, which represented post-replicative chromatin, decreased after 4 hours (from 60-70% to 30-40%). This applied both to early and late S phases.

I, however, found in dual labelling experiments (see Section 5.2.1) that within a 2-hour window (see Figure 5.2), patterns of replication foci tended to remain similar, with foci 2 hours apart often overlapping or adjacent to each other. After 6 hours, I however found that PML bodies were only half as associated with post-replicative chromatin. This may be due to the fact that post-replicative chromatin occupies a distinct nuclear position and pattern compared with the chromatin which is still replicating, Using dual labelling with this longer interval, it was evident that PML bodies associate with the foci that are replicating at time of fixation, rather than with foci that were replicating 6 hours prior to fixation.

These data indicate that PML bodies are dynamic and re-distribute to newly-formed replication foci throughout S phase. As the pattern of replication foci alters during S phase, this is mirrored in the changing pattern of PML bodies. This set of experiments also suggests that the proportion of PML bodies which associate with replicating DNA is not due to other unrelated factors (due to the limited volume of the nucleus), as the percentage of PML bodies associating with the foci would be constant.

5.2.4 Association of PML bodies with transcription foci in S phase

Kiesslich *et al* have shown that in unsynchronised cells a proportion of PML bodies associate (measured as number in direct contact) with transcription foci, as labelled with

flurouridine (Kiesslich *et al.*, 2002). Furthermore, cells arrested in G0 were noted to have a higher association with transcription foci, compared with unsynchronised cells. This suggests that in cells that are actively dividing, PML bodies associate less with transcription foci. This may be due to the fact that, during S phase specifically, PML bodies have decreased association with transcription foci. I therefore tested this possibility by comparing the association of PML bodies with transcription foci in cells specifically in S phase with the association in unsynchronised cells.

Transcription and replication foci were identified by simultaneously pulsing the cells with TR- dUTP for 1 hour (using the scratch-labelling method), and with fluorouridine for 15 minutes, prior to fixation. Fluorochrome-labelled dUTP was used instead of BrdU, since the primary antibody for fluorouridine was raised against BrdU and would therefore detect both transcription and replication foci. The fluorouridine-labelled transcription foci were then detected using a FITC-labelled secondary antibody, together with antibodies against PML bodies (Figure 5.7).

As scratch labelling does not label all cells in S phase, I could only compare S phase cells with unlabelled cells, which could be in all stages of the cell cycle including S phase. As in the previous section, the total number of PML bodies in each cell, and the proportion of PML bodies in direct contact with transcription foci were counted by two independent observers. I found that 35% of PML bodies associate with transcription foci in unlabelled cells, in agreement with a previous report (Kiesslich *et al.*, 2002), and 37% of PML bodies associate with transcription foci in cells in S phase (Table 5.2).

Thus, a similar proportion of PML bodies associate with transcription foci as with replication foci during S phase. The question thus arose as to whether PML bodies associate with either replication foci or transcription foci, but not both. However, visual examination of the cells labelled showed that individual PML bodies could associate with both replication foci and transcription foci, while some PML bodies do not associate with either replication or transcription foci. It should be noted that replication and transcription foci are reported to sometimes colocalise (Hassan *et al.*, 1994), or to be located adjacent to each other, occupying different "zones" (Ma *et al.*, 1998). My experiments would agree with the latter report by Berezney and colleagues, suggesting that replication and transcription foci do not often colocalise, regardless of the period

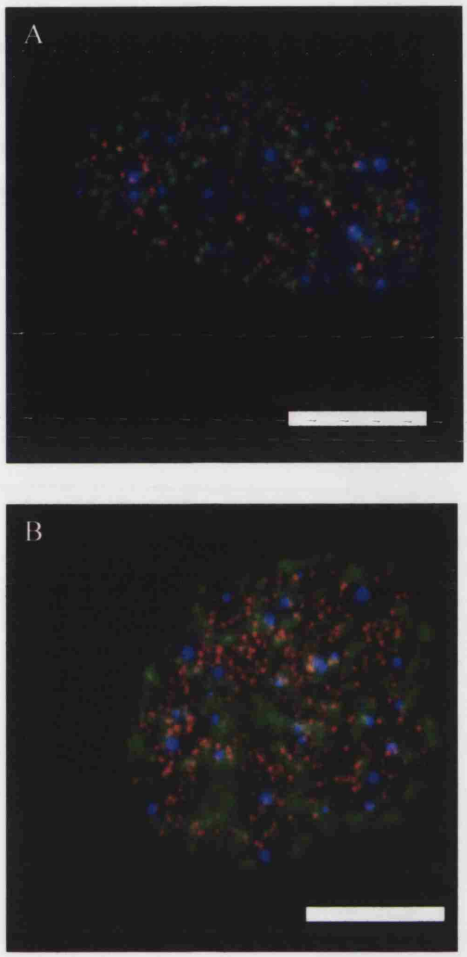

Figure 5.7. The association of PML bodies with replication and transcription foci. MRC5 cells labelled with PML bodies (blue), replication foci (green), transcription foci (red). A: cell in early S phase (S1). B: cell in late S phase (S3). Stacked images showing whole cell are shown. The solid line represents 5 micrometres. PML bodies are shown association with replication foci and transcription foci independently, although some PML bodies can associate with both replication and transcription foci.

Table 5.2. The number and proportion of PML bodies in contact with transcription foci under different conditions.

	Number of cells (n)	PML bodies (n)	PML/cell	PML/cell in contact	PML%
S-phase	43	1126	26.2	9.42	35.1
Unsynch	72	1030	14.3	5.03	37.3

within S phase, although they are frequently adjacent to each other (Figure 5.7.A and B).

It must also be noted that random associations may occur between PML bodies and the numerous transcription foci (or indeed between replication and transcription foci). I am unable therefore to exclude the possibility that the 30-40% association of PML bodies with transcription foci is due to chance. However, this percentage is consistent with that previously published (Kiesslich *et al.*, 2002). The statistical association of PML bodies with transcriptionally active genomic regions has already been studied in Chapter 3.

My observations also show that the association of PML bodies with replication or transcription foci is more complex. This is consistent with the view expressed so far in the thesis that the association of PML bodies with other nuclear or genomic regions does not necessarily reflect direct interactions but rather the level of transcriptional activity. To analyse this further, specific locus-PML associations were studied in the different stages of S phase.

5.2.5 Locus-PML association during S phase

I showed in Chapter 3 that for several loci along chromosome 6, there was no difference in their association with PML bodies between S and G0/1 phase cells, with the exception of the replication-dependent histone gene cluster, which is transcribed predominantly in S phase. This was determined using the statistical model that measured the mean minimum locus-PML body distances (mmd). To further confirm the association of PML bodies with regions of active DNA replication, immunoFISH was used to examine in detail three loci in S phase. These were: the *PBX2/NOTCH4* genes in the MHC class III region, which the laboratory has recently found to be early replicating (unpublished data); the TAP/LMP locus in MHC class II, which was later replicating; and the chromosome 6 centromere which as a heterochromatic region was expected to be one of the latest replicating sites. Unsynchronised MRC5 cells were labelled with BrdU, fixed for immunoFISH, and the various loci, PML bodies and replication foci visualised. Different populations of cells were then captured, based on their replication pattern, corresponding to cells in S1 or S2 (early replicating) and S3 or S4 (late replicating) (see Figure 5.8.A-B).

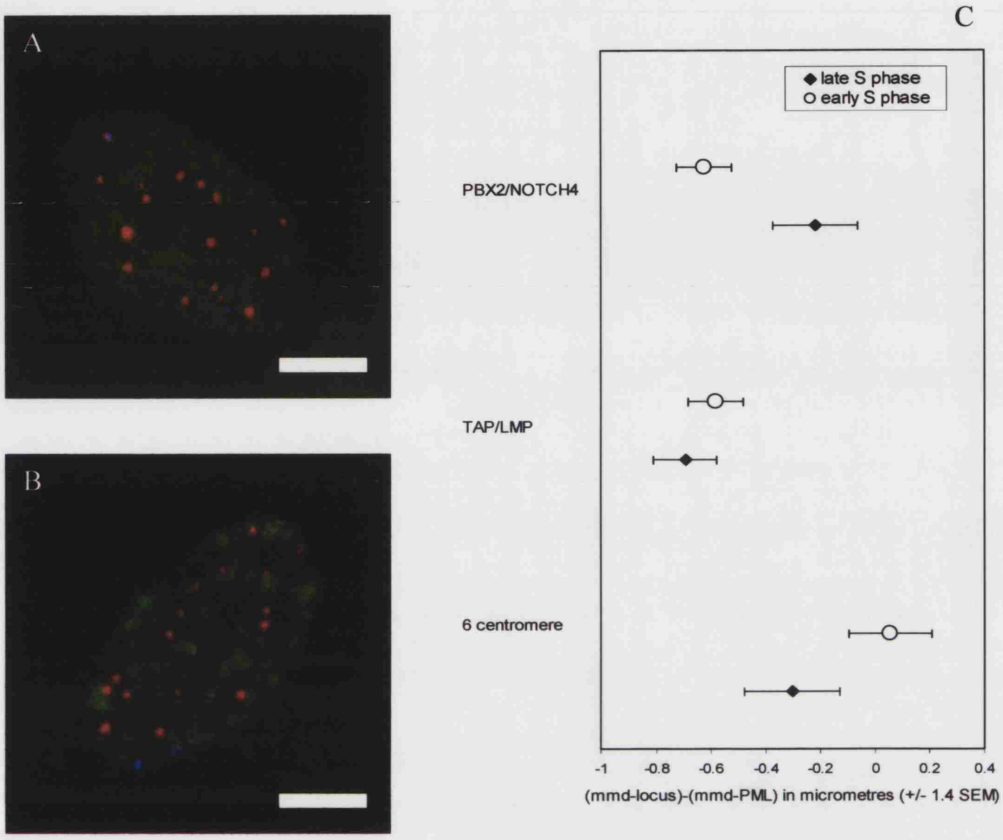

Figure 5.8. Association of PML bodies with loci on chromosome 6 in cells in early S phase compared with cells in late S phase.

A: MRC5 cell showing the PBX2/NOTCH4 gene (blue), PML bodies (red) and replication foci as labelled with BrdU (green). Cell is in early S phase (S2).

B: MRC5 cell showing the PBX2/NOTCH4 gene (blue), PML bodies (red) and replication foci as labelled with BrdU (green). Cell is in late S phase (S4).

C: Locus-PML distances for different loci were compared in cells in early S phase versus late S phase. The (mmd-locus) – (mmd-PML) on X-axis represents the distance to which a locus is further or closer to the nearest PML body compared with the PML-PML distance, with error bars showing 1.4 standard errors. Unpaired T-tests were performed between the cells of early and late S phases for the 3 loci. The PBX2 locus showed a significantly closer PML association in early S phase, while the chromosome 6 centromere showed the reverse.

The mmd associations were then compared using the Student's t-test, as in Chapter 3. For each locus, comparisons were made between cells in S1/S2 and those in S3/S4. I found that for the *PBX2/NOTCH4* locus, there was a statistically increased association with PML bodies in early S phase cells, compared with late S phase (Figure 5.8.C, Appendix C.9). In contrast, for the chromosome 6 centromere, there was an increased association with PML bodies in late S phase cells compared with early S phase. Finally, for the TAP/LMP locus, no statistical difference was seen between the early and late S phases.

These results show that the association of PML bodies with specific loci can change during different stages of S phase. Furthermore, they suggest that the highest locus-PML association occurs at the time of replication for each locus.

5.3 Discussion

There have been few studies examining the role of PML bodies in DNA replication, and these have been inconclusive (see Section 5.1.1). Yet, as I have noted, much circumstantial evidence exists as to such a role.

The findings from the previous chapters suggest an association between gene transcription and PML bodies. In this chapter, PML bodies were found to associate dynamically with replication foci throughout S phase, although quantification was only performed for S1, S3 and S4 periods of S phase. It was previously noted that some PML bodies lie adjacent to middle-late S phase replication foci, at least in one cell line (Grande *et al.*, 1996). I have confirmed this published observation. In addition, using the MRC5 primary fibroblast cell line, it was also possible to show that replication initiates with a few punctate foci, termed here as S1, compared with cancer cell lines that have a diffuse replication pattern in early S phase (Kennedy *et al.*, 2000). This S1 pattern allows for an association between PML bodies and early S phase foci to be quantified.

One concern with the method of assessment of association of PML bodies with replication foci by visual counting (as with transcription foci) is that the result may reflect a spatial phenomenon, as replication foci can occupy a large volume of the nucleus. However, the fact that the association of PML bodies with replication foci occurs dynamically throughout S phase would suggest otherwise. I also applied the

statistical method based on mmd distance measurements, and found temporally specific association of PML bodies with individual loci that were known or inferred to be either early or late replicating. These current observations may be thought to conflict with those in Chapter 3, where no increase (or decrease) of association was seen between G1/0 and S phases of the cell. It must be noted, however, that a locus that is actively replicating in a specific stage of S phase will show an association with PML bodies that differs in early or late S phases. For example, PML bodies were found to have a close association with the PBX2/NOTCH4 locus in early S phase, but to be further statistically from the locus in late S phase, such that the differences in mmd cancel each other out. Thus, the overall mmd distances in S phase would be statistically similar to those in G phase.

Even though I found that replication and transcription foci do not colocalise (Section 5.2.4), one concern for my results was that loci that were early replicating were also those that were highly expressed (see Section 5.1.3). The association with these regions may therefore reflect the transcriptional status of these sites. However, by means of dual replication labelling and washout experiments, it was noted that the association with nascent DNA from previously early replicating foci was dynamic and decreased with passage further into S phase. Since transcription of most genes is likely to continue over S phase, the association of PML bodies with these regions (which were replicating earlier, but not at the time of fixation) should persist if this association was due to transcriptional activity. Similarly, the association of PML bodies with centromeres was reported by Everett *et al* in S/G2 phases of the cell (Everett *et al.*, 1999a). My study showed that the association of PML bodies with late replicating foci did not persist after washouts of 4-8 hours. This result is compatible with the findings of Everett *et al*, as cells were expected to pass through mitosis after this period of time (Everett *et al.*, 1999a). The association of PML bodies with centromeres as previously observed may therefore suggest a role of PML bodies in replication of DNA at the centromeres.

5.3.1 Possible mechanism for association of PML bodies with replication sites

The fact that the association between PML bodies and nascent DNA decreases following washout experiments poses an interesting question. That is, does the newly formed DNA move away from the PML bodies, or are the PML bodies dynamic, moving (or reforming) away from completed replication sites and to new sites of DNA replication? These questions have already been partially addressed in Section 3.3.1,

where the possible mechanisms by which PML bodies associate with transcriptionally active genomic regions were discussed. However, several studies have shown dynamic behaviour of both nascent DNA and PML bodies specifically in S phase. Although PML bodies have previously been shown to be largely static in live cell experiments using GFP-tagged Sp100 and PML proteins (Muratani *et al.*, 2002; Wiesmeijer *et al.*, 2002), Eskiw *et al* found that PML bodies showed increased mobility related to an alteration of chromatin structure which is suggested to decrease the anchorage of PML bodies to the chromosome territories (Eskiw *et al.*, 2004). It is therefore possible that such chromatin changes may also occur during S phase (Bailis and Forsburg, 2003). This is consistent with my observation that some PML bodies in S phase are less rounded, and may even form cap-like structures around replication foci.

Live cell studies of GFP-labelled PCNA initially suggested that replication foci containing PCNA are relatively immobile structures (Leonhardt *et al.*, 2000). During the course of S phase, as the replication pattern changes, completed replication foci are dispersed, and new foci formed at adjacent sites. More recently, GFP-PCNA experiments were combined with microinjection of fluorochrome-labelled dUTP to label nascent DNA (Sadoni *et al.*, 2004). Using time-lapse microscopy, it was found that newly-replicated DNA moved away from replication foci, but only within <1μm in the local environment. Large-scale redistribution of DNA throughout the nucleus was not seen. Nevertheless, such magnitude of change may be sufficient to show a decrease in association between PML bodies and nascent DNA over time. My experiments using dual labelling suggest that over the course of the entire S phase, the nascent DNA from earlier periods of S phase (i.e. the post-replicative chromatin) retains the distinctive pattern for that period of S phase. Thus, the changes in the replication pattern between the different S-phase periods is more likely due to the formation of new replication foci rather than the movement of nascent DNA away from replication sites. This in turn suggest that PML bodies associate with sites of new replication foci, rather than the movement of post-replicative chromatin away from the bodies.

5.3.2 Possible basis for the association of PML bodies with replication sites

As in previous reports, the observations made here were largely descriptive. However, some of my observations enable possible functions to be suggested. Firstly, as with transcription foci, the association with replication foci appears to be indirect, since several replication foci were found close to PML bodies without direct contact.

Furthermore, although the number of PML bodies increased in S phase, this was not sufficient for colocalisation with all replication foci. Thus, as with transcription, PML bodies are not likely be directly involved in DNA replication at all sites. PML bodies may therefore relocate or form *de novo* in fertile domains for replication. The study with transcription and replication foci labelled simultaneously suggests that in S phase, PML bodies may retain a relationship to transcription domains. Thus, the function of PML bodies in replication may be *in addition* to that in transcription. This explains the observation that the number of PML bodies is cell cycle dependent, with the number rising throughout G1 phase, becoming much more numerous in S phase, and then decreasing in number in G2 phase (Koken *et al.*, 1995).

Although I have shown a spatial and temporal association between PML bodies and replication, there also appears to be evidence for a more direct role of PML bodies in DNA replication under specific circumstances. In one study of immortalised cell lines displaying 'alternative lengthening of telomerase' (ALT, see Section 1.3.7.6), PML bodies were found to associate with putative sites of telomere replication, as labelled with non-denatured BrdU foci in late S phase (Wu *et al.*, 2000). This observation was not, however, seen in another study using similar cell lines (Grobelny *et al.*, 2000). Furthermore, as mentioned above, PML bodies have been showed to be the site of viral DNA replication (as well as subsequent protein synthesis and virion packaging) (see Section 1.3.8.3). Furthermore, PML bodies have been shown to accumulate single-stranded DNA from viral replication (Jul-Larsen *et al.*, 2004). This may imply that under normal physiological conditions PML bodies serve to degrade excess or erroneous DNA fragments.

Since PML bodies have been suggested to act as a nuclear depot for many proteins, is there evidence that PML bodies contain any proteins used in replication? Unlike transcription, proteins directly related to DNA replication have rarely been documented to be associated with PML bodies. It must be noted that, as discussed in Chapter 4, the association of several transcription factors only became apparent with overexpression studies, and it may be that such experiments are required to determine if replication factors do colocalise with PML bodies.

One replication factor that is found in the replication complex, and in PML bodies is RPA (Wold and Kelly, 1988). RPA is involved in the ligation of the Okazaki fragments,

as well as in DNA repair (reviewed in MacNeill, 2001). RPA has been found in PML bodies in S phase but only in late stages (Bischof *et al.*, 2001; Yankiwski *et al.*, 2000). It has been proposed that RPA, together with BLM (also found at this stage) serves to repair mis-replicated DNA. In these studies, RPA has also been implicated in the ALT pathway (Section 1.3.7). The repair protein, RAD51, which has been shown to colocalise with PML bodies at repair sites, has also been shown to associate with post-replicative chromatin (Tashiro *et al.*, 2000). The different periods of S phase were not taken into account in this study. Furthermore, cells were not immuno-detected for PML, therefore it was not possible to determine if the RAD51-containing foci were in fact PML bodies. However, the washout period following the labelling of nascent DNA in that study was less than 1 hour. This timing is consistent with my washout time of 2 hours, when PML bodies were still highly associated with replication foci. As with RPA, the hypothesis in that study was that RAD51 foci served to repair mis-replicated DNA. This may then mirror the observation that PML bodies relocate to sites of DNA repair, together with repair proteins. In fact, many proteins are proposed to have dual roles in DNA repair and replication, such as PCNA, Mre11, NBS1 and Topors (a protein found to interact with Topoisomerase I) (see Section 1.2.3.3 and Bischof *et al.*, 2001; Rasheed *et al.*, 2002; Wu *et al.*, 2003a). These proteins have been found in PML bodies, but only in the context of DNA repair sites or ALT.

In addition to DNA replication itself, there is also evidence that PML bodies are related to cell cycle control, as discussed in Chapter 1. It may therefore be possible that such checkpoint functions are related to the positioning of PML bodies near replication sites, allowing origins to be triggered. However, this would only explain the association of PML bodies with early replication foci, rather than foci in S3 or S4.

In this chapter, I have examined the role of PML bodies with regard to the second major nuclear function after transcription. I found that PML bodies associate with replication foci throughout S phase. In addition, the association with different loci appears to be dynamic. However, as with transcription, the association with replication foci is not complete, and persists for a short period (up to 2 hours) after replication. This suggests that PML bodies act indirectly and in response to, rather than in the initiation of replication. They may therefore have a depot function, to remove excess of damaged DNA or replication machinery. Although predominantly descriptive, the association of

PML bodies with DNA replication sites is nevertheless significant, and should be studied further.

Chapter 6: Discussion

6.1 Summary

Despite huge advances in cell biology, the roles of most, if not all nuclear structures have not been fully understood. However, in the case of relatively well-characterised structures such as the nucleolus, the Cajal body, and speckles, only one dominant function has been found. This is not the case with the PML body. It is distinguished by the fact that many diverse nuclear processes have been linked with it.

Many studies have been performed in attempts to define a role of these bodies, which have involved a variety of *in vivo* and *in vitro* techniques. It is however important to distinguish whether a study focuses on the PML protein or the PML body. It may be that the PML protein in the nucleoplasm or cytoplasm may have different properties and functions to those in the nuclear bodies. A further possibility is that the PML protein in the bodies actually represents the inactive compartment of the protein. This situation is unlikely as PML protein is shown to be vital in recruiting other proteins to these bodies, suggesting an active or structural role here.

Another problem with the numerous studies published is to interpret whether its conclusions can be applied to cells in general, or only to a specific cell type or state of a cell. More importantly, since many studies have used transfection experiments, and these are potentially prone to artefacts (as seen with the experiments performed in Chapter 4), it is also important to understand what constitutes a physiological function (reviewed by Borden).

The overall aim of this thesis is to determine if there is a functional basis for the pattern and distribution of PML bodies. Descriptive experiments with microscopy techniques were predominantly used to characterise the distribution of PML bodies with respect to regions of nuclear activity. The advantage of this was that the behaviour of PML bodies can be studied in cells as close to physiological states as possible. ImmunoFISH experiments were performed to examine the association of PML bodies with individual loci. This was in contrast to other studies, in which PML bodies were detected in combination with general markers of transcription, such as nascent RNA or transcription factors. Such markers result in diffuse or microspeckled nuclear patterns,

which makes it difficult to exclude partial colocalisation due simply to the limited nuclear volume.

In this thesis, I have shown that PML bodies are located at nuclear regions of transcriptional activity, which is a function of both the gene-density and proportion of genes in the region which are active. Furthermore, transcriptional activity, rather than genetic composition is the key determinant of association, as shown by the association of PML bodies with genes on the active X chromosome, and the replication-dependent histone cluster on 6p22. Furthermore, apart from primary diploid fibroblasts, PML bodies have been shown to associated with the MHC in AHB lymphoblastoid cells (Shiels *et al.*, 2001), ATRA-treated NB4 promyelocytic leukaemic cells, renal carcinoma cell lines (CAKI-1 and CAKI-2), and HeLa ovarian carcinoma cell line (data not shown). Although the different cell lines may have different expressions of the classical MHC genes, SAGE analysis of a variety of cell lines have shown that many genes within the MHC cluster are highly expressed in a variety of normal and cancer cell lines (Caron *et al.*, 2001). Thus the MHC region is considered a RIDGE. Thus, the association of PML bodies with regions of transcriptional activity appears to be a common feature in a variety of cell lines.

In addition, the behaviour and distribution of PML bodies suggest a role that has been less explored, that is, in DNA replication. During S-phase, the number of PML bodies is known to increase (Koken *et al.*, 1995). In this thesis, it was shown that these additional PML bodies associate dynamically with foci of active DNA replication. This spatial association is also observed under physiological conditions. As with transcription foci, this association may be due to random factors, but the experiments using different time sequences, and the analysis of individual loci with different replication times excluded this possibility.

Despite the reservations with transfection experiments, it was recognised that such studies might allow a potential function to be confirmed. Therefore, to understand whether PML bodies directly affect transcription, both PML RNAi knockdown and overexpression studies were also performed. Both approaches were attempted, as they do not necessarily have opposite effects. Using RNAi knockdown experiments, I found that inhibition of PML expression did not appear to affect the genes that were associated with PML bodies. The only other study that uses RNAi to assess the role of PML bodies

likewise showed no effect on gene expression (Bruno *et al.*, 2003). In contrast, it was found that PML overexpression (or indeed control transfection) resulted in non-specific effect on the expression of both PML bodies and the MHC genes, as previously shown (Suzuki *et al.*, 1999; Tsukamoto *et al.*, 2000). While interpretations of overexpression experiments have to be made with caution, I nevertheless found by comparing PML transfection (compared with control transfection) that PML bodies do not appear to affect transcription of the genes studied.

I have also performed RNAi knockdown of MRC5 cells, and subsequently labelled with BrdU for cells in S phase (data not shown). It was noted that cells that had no detectable PML bodies in the nucleus were still able to enter into S phase. Furthermore, the overall proportion of cells in S phase was not markedly different from the control populations (both non-silencing RNAi treated and untreated) in contrast to previous studies (Mu *et al.*, 1997; Wang *et al.*, 1998a). Although I was not able to exclude subtle changes in the number of cells entering S phase, this result indicates that PML bodies are not required for DNA replication.

Can the results in this thesis be consistent with the available evidence already published? Roles in transcription and cell cycle control, as well as DNA repair, apoptosis and tumour suppression have been suggested for PML bodies. However, the key points to note are: (a) the PML protein occurs late in evolution, and knockout experiments of cell lines and mice show that the presence of PML bodies is not essential for life, and (b) PML bodies may facilitate or influence many nuclear processes, but do not appear to be crucial for these processes.

6.2 A possible integrated role of PML bodies

Based on these observations, I propose that in a normal cell population, the positions of most PML bodies are dependent on the transcriptional activity of nearby genes. I also propose that PML bodies form in response to surrounding transcription. I further propose that in S phase, additional bodies are formed in response to increased activity of the cell during DNA replication.

A unifying role for PML bodies would be that of a nuclear depot containing transcription factors and other proteins that alter the nuclear activities in response external stimuli (Negorev and Maul, 2001). Degradation of excess proteins may occur at

these sites as well (Fabunmi *et al.*, 2001; Mattson, 2000). This model supports the theory that PML bodies are regulatory structures, most probably acting to sequester or degrade excess protein factors in areas of accumulation (Anton *et al.*, 1999). From the experiments described in this thesis, this hypothesis may also be extended to other nuclear processes, such as DNA replication. I therefore propose that like its role in transcription, the PML body may act in response to sites of DNA replication, but acting to sequester or degrade excess factors or nascent DNA in these regions. Such a depot function means that PML bodies do not need to be formed in direct contact with transcription or replication foci, but only near denser regions of these activities. This is consistent with the fact that both nascent DNA and RNA do not associate directly or fully with PML bodies (Boisvert *et al.*, 2000; Grande *et al.*, 1996).

PML depots may also be aggregated at/near sites of excess, mutated or foreign protein accumulation (Tsukamoto *et al.*, 2000). At sites of DNA damage, various repair proteins are recruited to the aggregated PML bodies (Bischof *et al.*, 2001; Carbone *et al.*, 2002; Muto *et al.*, 2001). Yet another possible interaction may exist between PML bodies and nucleoli. Earlier studies have suggested that some proteins, such as p53 and BLM are found in both these nuclear structures (Yankiwski *et al.*, 2000; Zhang and Xiong, 1999). I have found that transcriptional inhibition (or RNA pol II activity) of MRC5 cells by α-amanitin or 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) treatment resulted in aggregation of PML bodies around the nucleus (data not shown). Although this is an artificial condition of the cell, it may suggest that a proportion of PML bodies interacts with the nucleoli, and this interaction becomes more prominent as the transcriptional activity in the rest of the nucleus is suppressed.

With these heterogeneous functions, it remains to be proved whether all PML bodies within a nucleus have the same constituent proteins, whether different PML bodies have different structures, and whether the function of each PML body can adapt to the requirements of the nucleus (Bloch *et al.*, 1999). PML bodies may even have different functions in different cell types, reflecting their varying levels of expression. For example, specific histological types have been shown not to express PML protein/bodies (see Section 1.3.7). It is debatable whether this is due to differential gene expression profiles, proliferation rates, or simply general cellular activities. The pattern and number of PML bodies may therefore reflect the overall requirements of the nucleus, as illustrated in Figure 6.1.

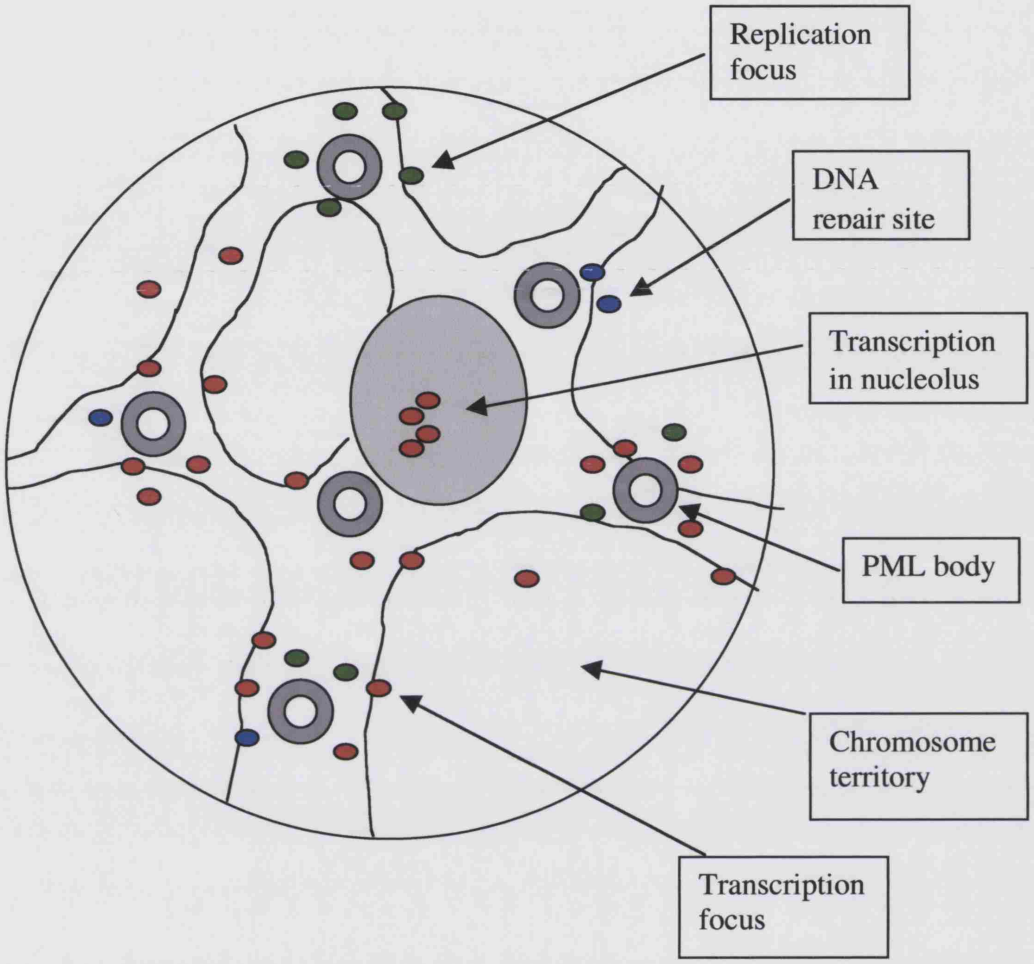

Figure 6.1. Proposed model of PML body function and position in the nucleus. PML bodies aggregate at regions of highest density/levels of transcription activity. Transcribed genes which are in regions with low overall transcription activity are less likely to associate with PML bodies. PML bodies may also associate with transcription activity in nucleoli. In addition, PML bodies associate with replication foci and repair sites. These bodies may be formed specifically for these sites.

In this thesis, I wished to understand the biology of PML bodies from the view of nuclear architecture. To this end, it is therefore suggested that PML bodies are dynamically distributed so that they function as depots for factors required for a variety of nuclear functions in the different nuclear regions.

6.3 Future Work

As mentioned, this thesis has taken a predominantly descriptive approach to examine PML bodies, although functional assays have been performed in the form of knockdown and overexpression studies. The justification of this approach is that the cell imaging technique used is important in understanding the functions of PML bodies in a more integrated and physiological manner. The future work that I would like to pursue includes (a) extension of my current experimental approach, and (b) novel and complementary approaches.

6.3.1 Improvements on current experiments

I used immunoFISH to analyse the spatial associations of PML bodies with specific loci. As discussed, this was found to be superior to visual association of PML bodies with transcription or replication foci, due to the spatial constraints of the nucleus. Furthermore, detection of transcription foci (by fluorouridine) may not necessarily reflect the sites of high transcriptional activity, but rather provide a snapshot of transcription within a narrow time window. Since the hypothesis is that PML bodies associated with sites of transcription or replication, it would be ideal if PML bodies can be found to associate with global markers of transcription (such as markers of CpG, Alu sequences or acetylated histone) and replication (which has already been done with BrdU, but not statistically). A more sophisticated statistical model could be set up whereby the intensities of various markers of activity around a certain distance from PML bodies could be measured. This might show that the surroundings of PML bodies have the highest concentration of transcriptional (or replicative) activity.

Another use of microscopy that I would like to pursue involves immunofluorescence to show colocalisation of PML bodies with different proteins. Pilot experiments were performed to co-detect PML bodies with (a) mediators of interferon action (e.g. STAT1, IRF-1 and CIITA proteins) and (b) replication factors (e.g. cyclins, cdk, ORC, MCM etc). These experiments have so far have been complicated by cross-reactions of the

primary or secondary antibodies, but would be useful in identifying the role of PML bodies in potential transcription pathways.

The use of RNAi treatment has been an invaluable tool in my work. Unfortunately, in the MRC5 cells used, the knockdown effect was limited to a maximum of 75%. I would therefore like to pursue the refining of the RNAi methodology. I would ideally like to perform a more effective knockdown effect, either by further siRNA oligonucleotide designs, the use of duplex hair-pin DNA inserts for more prolonged RNAi treatments (either by transient or stable transfections with inducible promoters), or the use of alternative cell lines. (The knockdown of PML bodies in HeLa cell line was also used, but the effect was again limited). The use of other cell lines would, however, require them to be tested for spatial gene-PML associations.

I have analysed the role of PML bodies in the interferon-mediated pathway. In particular, the CAKI-2 cell line, which is unresponsive to IFNγ has been used. I would ideally like to study this pathway further by other similar cell lines. Examples include cells from BLS patients, which are deficient in CIITA or RFX. It will be also useful to study PML bodies in the context of other pathways. These may include the TNFα, fos/jun (AP1) or Sp1 pathways. Rather than using overexpression methods as in published studies, the effect of treating cell lines with different ligands or cytokines, and combined with RNAi knockdown of PML, may be analysed. RNA-FISH for target genes of various pathways is also important to determine if they have a closer transcription-related association.

I have also not dismissed the use of PML overexpression methods. It may be that other models of transcription, other than the interferon pathway or the MHC, would be less prone to non-specific effects. Thus, in some of the above experiments, with correct compensation for the effects of transfection, the use of PML overexpression may have a role.

6.3.2 New methods and directions

Using the RNAi methodology, I also wish to confirm the effect PML bodies have on transcription. For this, two approaches to examine gene transcription would ideally be performed. Expression microarrays to allow genome-wide testing of changes in expression (although only in a crudely quantitative way) under RNAi treatment.

Alternatively, high-resolution analysis of all genes in a PML-associated region will be performed. For example, examination of all genes within the MHC or the localised chromosome region in band 1p36 or 9q34 will determine the role of PML bodies in these regions. This would involve large scale PCR, or alternatively, custom-designed array chips to cover a contiguous region.

To study post-transcriptional effects, I would like to perform RT-PCR analysis to examine ratios of nascent or pre-mRNA with intron-less mRNA levels of target genes. In addition, mRNA in cytoplasmic compared with nuclear fractions will be performed. This will allow me to study the effect of PML bodies (via RNAi treatment) on splicing and nuclear export. Furthermore, the protein analysis of different gene products by FACS and Western blotting will determine if ultimately the protein expression of a gene is altered.

Another technique that I have experimented with, but have yet to obtain reproducible results is the use of GFP-tagged PML and/or other proteins. I used GFP-PML transiently transfected into the MRC5 fibroblast cell line. To perform more flexible experiments, I would ideally like to construct stably expressing cell lines, with conditional or endogenous promoter regions. This would allow more long term and flexible live cell imaging. The area that I would like to apply this is the response of PML bodies to different inductions, such as interferon treatment. I would also like to visualise the behaviour of PML bodies during S-phase. Both these studies can be complemented by subsequent fixation and immunofluorescence to detect the association of PML bodies with sites of transcription or replication. This will enable me determine the dynamics of PML bodies in more physiological conditions.

The colocalisation studies that have been discussed, which attempt to isolate new PML-interacting protein, can also be complemented by co-immunoprecipitation studies. Cells under different conditions, such as interferon treated cell, cells treated with transcriptional inhibition, and cells in different phases of the cell cycle, can be harvested, and cell lysates subjected to immunoprecipitation with anti-PML antibodies. Various candidate proteins can then be tested by Western blotting analysis to determine if they associate with PML protein under different conditions.

A further extension of this approach is to analyse PML bodies by proteomic analysis. The use of large-scale proteomic studies has already been applied successfully to nucleoli and speckles. These studies depend on accurate isolation of PML bodies. Isolation of PML bodies biochemically using immunoprecipitation is possible, but this would not distinguish the PML protein also found in the nucleoplasm or cytoplasm. Alternatively, physical isolation of PML bodies may be technically difficult due to the size of these bodies, and their similarities to other structures. If a reliable method of PML body isolation can be performed, 2D protein gels and mass spectrometry can be performed to determine the protein composition of PML bodies. This would hopefully complete the list of PML-associated proteins, which at present appears to be an ever-expanding collection.

6.4 Conclusion

This thesis supports the growing belief that our elephant, the PML body, is a multifunctional nuclear structure. In addition, the findings in this thesis suggest that a higher order of nuclear regulation exists beyond that of discrete foci of activity (such as transcription or replication). That is, these foci, rather than being scattered throughout the nucleus, are spatially related to PML bodies.

My findings therefore subscribe to the increasing evidence that non-random arrangements exist between genes and nuclear structures. Overall, these findings contribute to the understanding of the nucleus as a highly organised structure. Although I have attempted to reveal the 'elephant' of the PML body, perhaps the real 'elephant' is the nucleus.

Appendix A: List of reagents

1 Cell culture

Phosphate-buffered saline A (PBS-A)
8g/l NaCl
0.2g/l KH2PO4
1.15g/l Na2HPO4
0.2g/l KCl

10% DMSO
1ml Dimethyl sulphoxide
9ml foetal calf serum

Bacterial grown and DNA extraction

LB-Broth
5 % Bacto-tryptone
1% NaCl
0.5% bacto yeast extract
pH 7.0

LB-Agar
LB broth with 1.5% agar
Solid media microwave-melted and cooled before addition of appropriate antibiotic

TE Buffer
10mM Tris.Cl pH 8.0
1mM EDTA pH 8.0

ImmunoFISH and Immunofluorescence

CSK buffer
10mM Pipes, pH6.8
0.1M NaCl
0.3M sucrose
3mM MgCl2
0.5% Triton-X solution (Sigma)

4% paraformaldehyde
10ml 16% methanol-free paraformaldehyde (TAAB labs)
30ml PBS-A

0.5% Triton-X permeabilisation buffer
250ul Triton-X solution (Sigma)
50ml PBS-A

20x SSC
3M NaCl
0.3M Sodium Citrate
pH 7.0

FISH hybridization buffer
50% deionized formamide
2 x SSC pH 7.0
10% dextran sulphate
2% Tween 20

Denaturation buffer
50% formamide
2 x SSC pH 7.0

SSCTM blocking buffer
4xSSC
0.5% Tween (Sigma)
5% Skimmed milk powder

TNB buffer
0.1M Tris-HCl, pH7.5
0.15M NaCl
0.5% BSA

TNT buffer
0.1M Tris-HCl, pH7.5
0.15M NaCl
0.05% Tween-20

RT-PCR and Agarose gel preparation

50xTAE
2M Tris
2M Glacial Acetic Acid
50mM EDTA

6x Loading Buffer
0.25% w/v bromophenol blue
0.25% w/v xylene cyanol
40% w/v sucrose

SDS-PAGE and immunoblotting

2x electrophoresis sample buffer
125mM Tris-HCl pH6.8
4% sodium-doc sulphate
10% glycerol
0.006% bromophenol blue
2% DTT

10X Running buffer
50.5g/l Tris-base
72g/l Glycine
0.1% sodium-doc sulphate

10X Transfer buffer
50.5g/l Tris-base
72g/l Glycine

Transfer buffer
50ml 10x transfer buffer
100ml methanol
make up to 500ml with distilled water

Appendix B: List of DNA probes and PCR primers.

Appendix B.1: Oligonucleotides used for RNAi and tranfection of PML.

SiRNA oligonucleotides

PML1 AATACAACGACAGCCCAGAAG

PML2 AAGTGAGGTCTTCCTGCCCAA

PML3 AAAGGCCCTTCCTATGGAGAG

PML4 AAGAGTCGGCCGACTTCTGGT

PCR primers for overexpression

Forward primer: CACCAAGCTGGGGTCCAT

Reverse primer: CTCTCAGAGGCCTGCTTGAC

Appendix B.2: BAC/PAC clones and PCR primers

List of genes analyzed with their BAC/PAC probes and primers designed against the corresponding mRNA sequences.

Locus	Cytogenetic band	Map data* (Mb)	BAC/PAC clone	Primer-Forward	Primer-Reverse	Tm (°C)	PCR size (bp)
GNB1	1 p36.21-33	1.1	RP1-140A9	TACACCACCAACAAGGTCCA	CAGTGTGTCCGGTAAACGTG	51.7/53.7	300
GNB1	1 p36.21-33	1.1	RP11-372I8				
PRKCZ	1 p36.2-33	1.5	RP4-592H21	GGAGCAGAACAGTCCCTCAC	AGGACGCTACACGCTCACTT	55.8/53.7	200
PRKCZ	1 p36.2-33	1.5	RP11-290B2				
TNFRSF14	1 p36.3-2	2	RP4-755G5	TCCTCTGCTGGAGTTCATCC	GGTACTCGTCCTCCTTGCAG	53.7/55.8	200
TNFRSF14	1 p36.3-2	2	RP11-285K17				
TNFRSF12	1 p36.2	5.7	RP4-666P17	CAGATGTTCTGGGTCCAGGT	GGAGGTGCTAGAAGGGTGTG	53.7/55.8	200
TNFRSF8	1 p36	11.3	RP5-1125M11	CCCAGAGAACTGCAAGGAAC	GGGACAGACCTGGATCTGAA	53.7/53.7	200
TNFRSF8	1 p36	11.3	RP11-426M1				
CASP9	1 p36.3-1	15.4	RP11-301D22	GTTGAGACCCTGGACGACAT	TCACTGGCAGAGAAAGAGCA	53.7/51.7	300
HMG17	1 p36.1	28.8	RP3-441F9	AAAGCAAAGGTGAAGGACGA	CCTGGTCTGTTTTGGCATCT	49.6/51.7	200
HMG17	1 p36.1	28.8	RP5-1080N1				
HMG17	1 p36.1	28.8	RP11-492M19				
EIF3S2	1 p34.1	33.5	RP1-286D6	GTCAACTCAGCTGCCCTCTC	ATCAGGATGGAAGGCAACAC	55.8/51.7	200
EIF3S2	1 p34.1	33.5	RP5-1199K24				

PSMB2	1 p34	36.4	RP11-374F9	GTCATCTGCTTCCTGCTTCC	TGCAGTGAGCCAAGATTACG	53.7/51.7	200
PSMB2	1 p34	36.4	RP11-435C22				
CRYZ	1 p31-22	78.6	RP11-369L17	CCCCGTGGAGACATACATTC	ACAGTGTGGTCTGCTGCAAG	53.7/53.7	200
CRYZ	1 p31-22	78.6	RP11-44I8				
NOTCH2	1 p12	147.4	RP5-1042I8	ACCCTTGTGAGAATGCTGCT	CCATACCACTGAAGCCTGGT	51.7/53.7	200
PSMB4	1 q21	152	RP11-247N18	GCACGAGGCTAAGATGGAAG	GAACTTAACGCCGAGGACTG	53.7/53.7	200
PSMB4	1 q21	152	RP11-431F23				
LMNA	1 p21	157.9	RP11-54H19	ATGGAGATGATCCCTTGCTG	CTTCTTCCCCAGTGGAGTTG	51.7/53.7	200
HSPA6	1 q21	158.6	RP5-822B19	CAAGATTCCCGAAGAGGACA	CTTGAGCGCTACAACTGCTG	51.7/53.7	200
HSPA6	1 q21	158.6	RP5-844J7				
CD1	1 q21-23	159	RP11-301C13	AGCCTGTATGGGTGAAGTGG	GTGTAGCTCCCACCCCAGTA	53.7/55.8	200
CD1	1 q21-23	159	RP11-404O13				
HF1	1 q32	200.7	RP11-347L19	CAGCAGTACCATGCCTCAGA	ACGGATGCATCTGGGAGTAG	53.7/53.7	200
HF1	1 q32	200.7	RP1-15D12				
ITPKB	1 q41-43	217.2	RP11-115P16	GGACCTACCTGGAGGAGGAG	TGATTCCCTCGATCCTGAAC	57.8/51.7	200
PSMB5	14 q11		RP11-68M15	CCATACCTGCTAGGCACCAT	CCTCTCTTATCCCAGCCACA	53.7/53.7	200
PSME1	14 q11.2		RP11-256C2	AGAAGAAGGGGGAGGATGAA	CTTCTCCTGGACAGCCACTC	51.7/55.8	200
PSME2	14 q11.2		RP11-256C2	GGAAGCTTTCCAGACAACCA	CCAGGTTGCTGCTGATGATA	51.7/51.7	200
PSMB10	16 q22		RP11-331B16	GGGCTTCTCCTTCGAGAACT	TGGATCTTCTCGCAGCTCTT	53.7/51.7	200
PSMB10	16 q22		RP11-510L21				

COL1A1	17 q21.3		RP11-167M14	CTCCCCAGAAGACACAGGAA	TCAAAAACGAAGGGGAGATG	53.7/49.6	200
TGIF	18 p11.3		RP11-113J12	GATAGCGGCCAAAAACTTCA	TTGCCTGAAGCTCCATCTCT	49.6/51.7	200
LMAN1	18 q21-22		RP11-4G8	GAATTGGAGCTGATGGCCTA	GACTAGCCCCGTCATTTTGA	51.7/51.7	200
C3	19 p13.3-13.2		RP11-383B15	CTCCGATAGGAACACCCTCA	GCTTGTTCAGCTTTCCATCC	53.7/51.7	200
KIR2DL3	19 q13.4		RP1-1060P11	ATCCCTTTCACCATCCTCCT	TCTGAGAAGGGCGAGTGATT	51.7/51.7	200
TFAP2	6p24		(see text)	GATCCTCGCAGGGACTACAG	TTGCTGTTGGACTTGGACAG	55.8/51.7	200
HMG17 L3	6 p22		RP11-457M11	TACCATGCCCAAGAGAAAGG	TCCTTTCCAGCATCTGCTTT	51.7/49.6	200
HMG17 L3	6 p22		RP11-318I12				
C4B	6 p21.3		(see text)	AGATCACCCAAGTCCTGCAC	CTCCTCGATCCAGCTATTCG	53.7/53.7	200
HLA-A	6 p21.3		(see text)	GTATTGGGACCGGAACACAC	GATGTAATCCTTGCCGTCGT	53.7/51.7	200
LMP2	6 p21.3		(see text)	GGCCAGAGCTGACTGTCAGT	CCTGTAGGAGCCTCATCTCC	55.8/55.8	200
ACTB	7 p13		RP11-90J23	AAAAGCCACCCCACTTCTCT	CTCAAGTTGGGGGACAAAAA	51.7/49.6	200
LAMB1	7 q22		RP11-77E2	CCAAACTGCAGAACTGACGA	CAGCACTTTGTTTTGCCTCA	51.7/49.6	200
INSL4	9 p24	1.9	RP11-140C18	AAGGCTGAGAACACCCAGAA	GGGTGGTGGTGAATGTCTTC	51.7/53.7	200
TYRP1	9 p23	9.3	RP11-214I20	CACGCCTCCTTTTTATTCCA	TCTGTGAAGGTGTGCAGGAG	49.6/53.7	200
IFNA1	9 p22	24.6	RP11-1P8	ATGGCAACCAGTTCCAGAAG	GGAGTTTCTCCCACCCTCTC	51.7/55.8	200
IFNW1	9 p22	24.6	RP11-1P8	CCTGGAACATGACCCTCCTA	TTTCAAGATGAGCCCAGGTC	53.7/51.7	300
TEK	9 p21	28.7	RP11-57P14	ACAATGGTGTCTGCCATGAA	ATCCTTGGAAGCGATCACAC	49.6/51.7	300
TEK	9 p21	28.7	RP11-152A20				

TEK	9 p21	28.7	RP11-183K1				
PRSS4	9 p13	34.3	RP11-133O22	CACTCTGAGCTTTGGTGCTG	GCAGCGATGGTGTCCTTAAT	53.7/51.7	300
PAX5	9 p13	37.2	RP11-12P15	CTATGAGCTTCTCCCGATGG	GCCCACAGAAAAGCAAGAAG	53.7/51.7	200
CREB3	9 p13	39.1	RP11-112J3	CTCCCCATCTGTGTCCAAAT	GTTCCCTGACTGCGTGAAAT	51.7/51.7	200
CREB3	9 p13	39.1	RP11-327L3				
TRKB	9 q22	76.9	RP11-263K15	TCCATCTCCCTTGGTTGTTC	TGTTAGTTGTGGTGGGCAAA	51.7/49.6	200
TMOD	9 q22.3	89.2	RP11-244N9	GGATGAGCTGGACCCTGATA	ATCGCTGCAATGTCACAGAG	53.7/51.7	300
TMOD	9 q22.3	89.2	RP11-24E19				
PSMD5	9 q31-34	114.2	RP11-27I1	CCAAGATGGGCAAATCACTT	GTGCAGTGGCAAGATCTCAG	49.6/53.7	200
C5	9 q34	115.8	RP11-556L12	CTGTTGAAGCCCGAGAGAAC	ATCTCCCAGGGAAAGAGCAT	53.7/51.7	200
PSMB7	9 q34.11-12	118.5	RP11-101K10	GCAACTGAAGGGATGGTTGT	ATCTGCTTCAGCATCCGATT	51.7/49.6	200
PSMB7	9 q34.11-12	118.5	RP11-121A14				
HSPA5	9 q33-34	119.4	RP11-65N13	GGTGAAAGACCCCTGACAAA	GTCAGGCGATTCTGGTCATT	51.7/51.7	200
HSPA5	9 q33-34	119.4	RP11-233N7				
PBX3	9 q34	120	RP11-282P20	TGGATACCCTCCGTCATGTT	AGGAAAAGTGTGGCCAGAGA	51.7/51.7	200
PBX3	9 q34	120	RP11-374O2				
RXRA	9 q34.3	129.4	RP11-92B21	CCTTTCTCGGTCATCAGCTC	CTTGGTGAAGGAAGCCATGT	53.7/51.7	200
ABC2	9 q34	131.4	RP11-229P13	GAGATCCGCAGAGAGATGGA	CTTCAGGATGAGGTCCCAGA	53.7/53.7	200
NOTCH1	9 q34	131.7	RP11-370H5	ACTGTGAGGACCTGGTGGAC	TTGTAGGTGTTGGGGAGGTC	55.8/53.7	200
NOTCH1	9 q34	131.7	RP11-413M3				

ALTE	X p22.33	1.1	RP11-325D5	CCTGTCCTACCACCTGGAGA	GTCAGCTCCTGCTGCTTCTT	55.8/53.7	200
DMD	X p21.2	29	RP11-46A23	AGTCAGCCACACAACGACTG	CCTTGGCAACATTTCCACTT	53.7/49.6	200
USP9X	X p11.4	38	RP11-299D7	TCAGGATGTGGGTCGTTACA	TGTCTGCCAAGCCTTTTCTT	51.7/49.6	200
USP9X	X p11.4	38	RP11-469E19				
XIST	X q13.2	69	RP13-216E22	TTACAGCAGGGGGTACTTGG	AGGGAAGTGAGTGGGGTCTT	53.7/53.7	200
NAP1L3	X q21-22	89	RP1-32F7	GGGGATGGGAAATTGAAGAT	TCTTCTCGTGGTTCCAGCTT	49.6/51.7	200
NAP1L3	X q21-22	89	RP1-60G11				
GLA	X q22	96.6	RP1-164F3	AGCCTGGGCTGTAGCTATGA	TGCCTGTGGGATTTATGTGA	53.7/49.6	200
PSMD10	X q22	104	RP5-889N15	AGCAGCCAAGGGTAACTTGA	CACTTGCAGGGGTGTCTTTT	51.7/51.7	200
PSMD10	X q22	104	RP11-448E12				
LAMP2	X q24	115	RP1-318C15	CGTTCTGGTCTGCCTAGTCC	ATCATCCCCACAAATGCTTC	55.8/49.6	200
UBE2A	X q24-25	116	RP5-876A24	CGGGAATATGAAAAGCGTGT	TTGGCAAGGAAGATGGAAAC	49.6/49.6	200
G6PD	X q28	149	RP5-1087L19	CTGTTCCGTGAGGACCAGAT	GGATGATCCCAAATTCATCG	53.7/49.6	200

* Mapping location (by NCBI BAC resource web-page or Sanger Ensemble web-page), are only shown for chromosomes 1, 9 and X loci. Values are in megabases from the p arm telomere.

Table B.3: List of genes analyzed quantitative real-time PCR with primers designed against the corresponding mRNA sequences.

Gene	Primer – Forward *	Primer – Reverse *
ABC2	CGACTGCACCGGTATGGG	GTGCCAAATGAGGCTGGG
NOTCH1	AACGCTTCAGACGGTGCC	AACCGGAACTTCTTGGTCTCC
LMP2	ACCGGGACCACCATCATG	AGCTTGTCAAACACTCGGTTCA
HLA-DRA	GTCTGGCGGCTTGAAGAATTT	GGTTGGCTTTGTCCACAGCTA
COL1A1	CGAAGACATCCCACCAATCAC	CACGTCATCGCACAACACC
PSMB10	AGCCCGTGAAGAGGTCTGG	CATAGCCTGCACAGTTTCCTCC
PSMB7	CAGGTATCAAGGTTACATTGGTGC	GGTCCAGTAACATCTACTCCCCC
PSMD5	TTTACGGCCATTGCAAACC	GTCATGCTCCACAGACCGG
HSPA5	GTTATGAGGATCATCAACGAGCC	CACATCGAAGGTTCCGCC
ACTB	ACCATTGGCAATGAGCGG	CCACAGGACTCCATGCCC
EIF3S2	GAAACAGGAAAGCAGCTGGC	AGCCCATCTGCTTGTCCG
PSMB4	GAGTGCTGTACTACCGAGATGCC	GTGGCGATTTGAAACCGG
CRYZ	ACAAGGAGCTGCCATCGG	CTCTCCAGCTTTCACACAGGC

* Forward and reverse primers were designed with annealing temperatures (Tm) between 58 and 60°C, and with PCR products between 50 and 150 bp.

Appendix C.1. Analysis of associations of different loci with PML bodies (mmd-locus), with closest associations shown first.
Measurements for the TAP/LMP locus are pooled from all sets of data in the table, where TAP/LMP is detected together with another locus in 3-color experiments. The loci shaded light grey have significantly closer locus-PML associations compared with the TAP/LMP locus.The loci shaded dark grey have significantly further locus-PML associations compared with the TAP/LMP locus. Local transcriptional activity calculated within +/- 1 cR of the locus. Local gene density is given as number of genes +/- 500 kb of locus.Transcriptional activity of each gene assessed by RT-PCR, relative to the β-actin gene (=1).

Locus	cells (n)	Average no of PML bodies	mmd-locus [1] (μm)	mmd-TAP/LMP [2] (μm)	mmd-PML [3] (μm)	T score (paired, vs TAP/LMP)	p value	% of loci in contact with PML	% of loci with md <1um	Local transcriptional activity	Local gene density	Transcription of individual gene
ABC2	29	10	1.30	1.63	2.39	-2.48	0.02	22.8	32.8	271	70	0.96
HMG17	30	12.8	1.37	1.65	1.98	-3.14	<0.01	21.9	33.8	235	47	3.49
HSPA5	33	14.7	1.37	1.48	2.04	-1.31	0.20	17.7	28.8	408	23	5.20
NOTCH1	31	10.3	1.38	1.68	2.30	-2.54	0.02	23.4	27.4	339	79	0.04
PBX3	34	12.8	1.44	1.59	2.01	-1.43	0.16	16.7	25.0	381	10	4.73
COL1A1	30	12.8	1.54	1.57	2.06	-0.34	0.73	17.7	11.7	259	47	1.40
C3	35	11.4	1.55	1.79	2.39	-1.99	0.05	22.2	24.3	135	62	0.72
PSMB10	39	10.6	1.55	1.66	2.20	-0.81	0.42	27.5	29.5	985	77	0.84
PSMB7	30	15.8	1.55	1.57	2.17	-0.09	0.93	16.6	25.0	179	23	3.29
KIR2DL3	34	13.4	1.56	1.49	2.01	0.49	0.63	18.5	23.4	229	69	0.00
RXRA	51	15	1.56	1.73	2.31	-1.66	0.10	20.3	20.6	103	29	0.90
PSMD5	35	10.3	1.63	1.69	2.23	-0.62	0.54	13.5	21.4	8	18	0.58
PAX5	30	11.3	1.66	1.74	2.33	-0.89	0.37	23.3	13.3	48	27	0.39
TAP/LMP	1841	12	1.66	-	2.25	1	1.00	17.3	19.5	1181	70	0.21
PRKCZ	30	14	1.67	1.57	2.10	0.74	0.47	12.5	18.3	559	47	1.57
CREB3	31	12.7	1.68	1.88	2.35	-1.48	0.15	25.0	25.8	23	57	1.23
GNB1	49	12.6	1.68	1.65	2.16	0.32	0.75	11.5	16.7	559	61	2.67
LMNA	32	11.2	1.71	1.67	2.15	0.32	0.75	18.8	17.2	137	78	1.64
PRSS4	34	10.7	1.71	1.72	2.24	-0.1	0.92	16.2	13.2	60	39	1.80
NOTCH2	61	12.6	1.75	1.6	2.18	1.72	0.09	19.7	17.2	4	36	2.13
ITPKB	34	12.3	1.77	1.55	2.42	1.8	0.08	10.0	17.7	144	23	0.32
9cen	32	9.7	1.80	1.72	2.34	0.056	0.58	25.4	21.9	0	0	0.00
HLA-A	28	12.8	1.80	1.59	2.25	2.62	0.01	21.7	17.9	333	51	0.00
C5	31	9.9	1.81	1.60	2.29	1.69	0.10	17.2	21.0	58	28	0.89

IFNA1/W1	70	10.3	1.81	1.69	2.34	1.27	0.21	13.5	12.2	126	39	0.64
LMAN1	36	12.5	1.81	1.81	2.44	0.0008	1.00	11.1	12.5	10	25	3.91
LAMB1	38	10.5	1.82	1.66	2.27	1.34	0.19	16.7	12.5	158	27	4.27
6p24	35	18.1	1.84	1.53	2.09	3.04	<0.01	12.3	8.6	63	26	0.48
PSMB5	32	11.3	1.84	1.87	2.40	-0.18	0.86	15.2	7.8	283	65	2.58
C4	32	12.6	1.85	1.90	2.33	-1.27	0.21	8.6	17.2	727	146	0.00
PSME1/2	31	11.3	1.86	1.74	2.30	1	0.32	16.4	14.5	322	70	3.70
INSL4	35	11.3	1.88	1.74	2.41	0.93	0.36	17.6	11.4	3	32	0.10
TMOD	35	10.5	1.91	1.73	2.37	1.72	0.09	15.7	10.0	20	24	1.38
19cen	32	11.7	1.93	1.46	2.27	2.72	0.01	13.5	10.9	0	0	0.00
TNFRSF8	32	10.2	1.94	1.60	2.07	2.94	<0.01	9.8	9.4	106	46	0.14
CASP9	31	11.1	1.96	1.60	2.24	2.54	0.02	13.3	9.7	72	42	1.71
EIF3S2	31	15.3	1.96	1.54	2.09	3.55	<0.01	16.4	19.4	89	45	1.12
PSMB2	32	12.6	1.97	1.41	2.06	4.88	<0.01	10.0	12.5	97	43	0.27
TYRP1	33	12.3	1.97	1.54	2.42	4.28	<0.01	12.9	4.6	6	11	0.00
TNFRSF12	34	10.2	1.98	1.62	2.22	2.65	0.01	10.4	14.7	26	41	0.42
TEK	32	10.4	1.99	1.63	2.30	2.77	<0.01	6.3	4.7	20	22	1.86
TGIF	36	12.6	2.01	1.69	2.26	2.34	0.03	15.3	11.1	30	22	1.49
1cen	31	10.2	2.03	1.62	2.27	2.36	0.03	17.8	11.3	0	0	0.00
TNFRSF14	32	13	2.04	1.59	2.25	3.73	<0.01	3.9	17.2	202	35	0.59
HMG17L3	32	13.7	2.07	1.65	2.18	4.16	<0.01	7.8	14.1	28	104	2.57
HSPA6	34	9.9	2.07	1.70	2.26	2.42	0.02	8.8	4.4	59	27	0.15
18cen	31	13.9	2.11	1.64	2.30	3.95	<0.01	20.6	6.5	0	0	0.00
6cen	29	12.3	2.14	1.61	2.10	6.49	<0.01	10.6	5.2	0	0	0.00
CD1	35	11	2.14	1.70	2.21	3.16	<0.01	8.9	5.7	72	40	0.32
ACTB	33	12.8	2.16	1.58	2.08	4.5	<0.01	5.9	1.5	55	35	1.00
HF1	31	12.9	2.16	1.84	2.34	2.33	0.03	12.1	6.5	0	24	2.65
PSMB4	65	11.4	2.17	1.74	2.25	4.43	<0.01	10.0	7.7	73	54	1.76
TRKB	33	9.9	2.22	1.61	2.26	5.92	<0.01	6.9	3.0	0	7	0.23
CRYZ	35	11.6	2.27	1.81	2.42	4.01	<0.01	8.6	5.7	37	13	2.66

[1]mmd-locus = the mean minimum distance from the loci (described in the first column) to the nearest PML body in a set of cells.
[2]mmd-TAP/LMP = the mean minimum distance from the TAP/LMP loci to the nearest PML body in the set of cells in which the TAP/LMP locus were detected at the same time as the locus described in the first column.
[3]mmd-PML = the mean minimum distance from each PML body to the nearest neighbouring PML body in the same set of cells.

Appendix C.2. Comparison of different loci in before and after signals in contact with PML bodies were removed from the set.

	Before removal					After removal					
Locus	Number of loci (n)	mmd-locus (μm)	mmd-TAP/LMP (μm)	t score (unpaired)	p value	Number of loci (n)	Number of TAP/LMP loci (n)	mmd-locus (μm)	mmd-TAP/LMP (μm)	t score (unpaired)	p value
NOTCH1	62	1.38	1.68	2.34	0.01	45	49	1.66	1.93	2.21	0.01
RXRa	102	1.56	1.73	1.62	0.05	81	89	1.78	1.88	0.95	0.17
IFNA1	140	1.80	1.69	-1.22	0.11	123	114	1.94	1.91	-0.36	0.36
NOTCH2	122	1.75	1.60	-1.59	0.06	101	98	1.94	1.80	-1.36	0.09
TGIF	72	2.01	1.69	-2.57	0.001	64	63	2.18	1.83	-2.96	0.001
TRKB	66	2.22	1.61	-4.17	0	64	50	2.26	1.88	-2.55	0
PSMB4	120	2.16	1.73	-4.30	0.001	120	110	2.27	1.90	-3.84	0.001

Appendix C.3. Summary of Student's t-test (paired) for the genes studied along chromosome X, the gene density and transcriptional activity around each gene calculated according to the text, and the transcription level of each gene calculated by RT-PCR.

Locus	cells (n)	Average no of PML bodies	mmd-active (μm)	mmd-inactive (μm)	mmd-PML (μm)	T value (paired, active vs inactive)	p score (active vs inactive)	Local Gene Density	Transcription of individual gene
ALTE	46	13.7	2.19	2.20	2.51	0.04	0.97	18	0.88
DMD	43	17.7	2.27	2.28	2.31	0.20	0.83	1	2.93
USP9X	42	15.3	1.84	1.93	2.35	0.65	0.69	19	1.48
Xcen	63	15.3	2.02	2.29	2.29	1.75	0.08	0	0
XIST	44	13.6	2.07	2.16	2.38	0.44	0.65	14	0.53
NAP1L3	38	15.4	1.90	2.42	2.40	2.52	0.01	9	0
PSMD10	42	12.1	1.68	2.02	2.28	2.61	0.01	12	0.79
GLA	43	13.8	1.74	2.16	2.48	3.25	0	28	2.01
UBE2A	57	12.5	1.91	2.38	2.59	2.71	0.01	24	1.2
LAMP2	51	14.7	2.16	2.60	2.42	2.14	0.04	27	1.3
G6PD	51	13.2	1.88	2.31	2.42	2.26	0.03	41	2.52

Appendix C.4. Comparison of different loci in cells during G0/1 phase versus S phase for MRC5 fibroblast cells.

	G0/1 phase cells					S phase cells						
Locus	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	t score (unpaired)	p value
6cen	63	13.1	2.21	2.46	-0.24	63	17.3	2.13	2.31	-0.18	0.51	0.61
TAP/LMP	60	11.1	1.66	2.31	-0.65	62	16.4	1.46	2.12	-0.66	0.06	0.95
Histone	66	11.1	2.04	2.23	-0.19	63	17.1	1.59	2.05	-0.46	2.41	0.02
6p24	59	11.4	2.44	2.52	-0.14	62	15.3	1.96	2.36	-0.26	0.80	0.43

Appendix C.5. Comparing the locus-PML association with the location of the locus signal relative to the chromosome territory.

	md-locus (um)			
	External signals	Peripheral signals	Internal signals	Significance (regression)
TAP/LMP	1.56um	1.56um	1.68um	P=0.76
ABC2	1.4um	1.8um	1.4um	P=0.28
HMG17	1.01um	1.42um	1.4um	P=0.54
Total	1.44um	1.60um	1.51um	P=0.57

Appendix C.6. Comparison of different loci in NB4 cells treated with ATRA.

Locus	cells (n)	Average no of PML bodies	mmd-(locus) (μm)	mmd-(TAP/LMP) (μm)	mmd-PML (μm)	T value (paired, TAP/LMP vs locus)	p score (TAP/LMP vs locus)
6p24	30	15.5	2.41	1.88	2.41	-7.04	0.00
6cen	31	16.9	2.65	1.92	2.57	-8.18	0.00

Appendix C.7. Comparison of different loci in cells treated with IFNγ versus untreated MRC5 fibroblast cells.

			Untreated cells					IFNγ treated				
Locus	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	t score (unpaired)	p value
6cen	29	12.3	2.14	2.10	0.04	31	15.3	1.87	1.96	-0.09	-0.94	0.35
6p24	35	16.6	1.84	2.09	-0.25	32	18.1	1.82	1.89	-0.07	1.47	0.15
TAP/LMP	69	12.9	1.62	2.19	-0.57	63	15.6	1.2	1.73	-0.53	0.41	0.68
PSMB7	30	15.8	1.55	2.17	-0.62	63	15.6	1.17	1.73	-0.57	0.35	0.73
PSMB10	39	10.6	1.55	2.20	-0.65	63	15.6	1.17	1.73	-0.56	0.61	0.54

Appendix C.8. Comparison of different loci in cells treated with IFNγ versus untreated, for the CAKI-1 and –2 cell lines.

Cell Line				Untreated cells					IFNγ treated				
	Locus	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	t score (unpaired)	p value
CAKI-1	TAP/LMP	30	28.4	1.58	2.31	-0.73	30	30.2	1.42	2.15	-0.73	0.02	0.98
	6 cen	30	28.4	2.21	2.31	-0.10	30	30.2	2.24	2.15	0.09	-1.59	0.12
CAKI-2	TAP/LMP	31	32.5	1.68	2.34	-0.66	33	31.6	1.53	2.18	-0.65	0.22	0.82
	6 cen	31	32.5	2.36	2.34	0.02	33	31.6	2.31	2.18	0.13	0.90	0.37

Appendix C.9. Comparison of different loci in cells during early versus late S phase for MRC5 fibroblast cells.

			Early S phase cells					Late S phase cells				
Locus	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	cells (n)	Average no of PML bodies	mmd-locus (μm)	mmd-PML (μm)	(mmd-locus) –(mmd-PML) (μm)	t score (unpaired)	p value
6cen	49	16.3	2.43	2.37	0.06	49	14.2	2.23	2.54	-0.30	2.18	0.03
TAP/LMP	49	15.7	1.59	2.17	-0.58	35	13.9	1.59	2.28	-0.69	1.00	0.31
PBX1	40	16.8	1.60	2.22	-0.63	41	16.1	2.14	2.36	-0.22	-3.29	0.001

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